ABSTRACT

MACKENZIE, ELIZABETH LOUISE. Regulation of Ferritin H in the Cellular Response to Stress. (Under the direction of Yoshiaki Tsuji.)

Cells are exposed to a constant onslaught of diverse stressors from exogenous and endogenous sources. Due to the ubiquitous nature of such insults, cells have evolved elaborate systems of defense. Phase II genes encode enzymes that are responsible for the reduction in toxicity of xenobiotics and other stressors and for cytoprotection. Ferritin H is one such phase II gene that protects the cell from oxidative stress by tightly regulating the levels of free intracellular iron that are available to catalyze the formation of potent reactive oxygen species (ROS). The purpose of this research was to elucidate the mechanisms of ferritin H regulation in response to oxidative stress that results from mitochondrial complex I inhibition by rotenone, and also from elevated intracellular calcium concentrations.

Rotenone induced transcription of the ferritin H gene though oxidative stress-mediated activation of an antioxidant responsive element (ARE). Binding of AP-1 family transcription factors to the ferritin H ARE was enhanced by rotenone treatment. Oxidative stress was required for ferritin H induction, demonstrated by the ability of the glutathione precursor and antioxidant, N-acetyl-L-cysteine, to abrogate rotenone mediated upregulation of ferritin H. In addition, reduced ferritin H expression by siRNA enhanced ROS production and sensitized cells to rotenone-induced apoptosis, suggesting a role for ferritin H in cellular protection from oxidative stress and cell death resulting from mitochondrial dysfunction.

Increased intracellular calcium concentrations and ROS are concordantly produced in activated Jurkat T-cells. Calcium ionophore induced ferritin H mRNA and protein expression; however, calcium did not activate ferritin H transcription. In addition, induction of ferritin H was independent of activation of Nuclear Factor of Activated T-cells (NFAT). Interestingly, elevated calcium led to retarded degradation of ferritin H mRNA, suggesting mRNA stability was enhanced. In addition, an au-rich element, which is a putative target for RNA binding proteins involved in regulation of mRNA stability, was identified in the 3'untranslated region of ferritin H mRNA. Taken together, these results indicate that ferritin H induction by calcium is regulated through a posttranscriptional mechanism involving mRNA stabilization.

Elucidation of mechanisms of ferritin H regulation by stressors, such as ROS and calcium, may offer insight into the cellular response to stress, and has potential implications in chemoprevention of disorders caused by calcium or ROS mediated apoptosis and cell damage, including aging, neurodegeneration, and cancer.

REGULATION OF FERRITIN H IN THE CELLULAR RESPONSE TO STRESS

by

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DEDICATION

I dedicate this work to my mom, who has filled my life with unconditional love, frequent phone calls, and unwavering support. She is my touchstone.

And to Tom, who taught me that dreams do come true, and helped me to realize mine.

BIOGRAPHY

It was early morning on Tuesday, July 24th, 1979 when Elizabeth Louise MacKenzie, the daughter of Lisa Edna Fives and Jonathan Mackenzie, and younger sister to three year-old Sarah Kate, entered the world. From the beginning, she was called 'Libby', and was the only girl, surrounded by boys, in the maternity ward. Ironically, she would spend her graduate career in very much the same situation. Her childhood included a propensity for hysterical laughter at an extremely early age, much to the bewildered astonishment of her parents, who asked eachother, "Is that the baby?" Each night her mother, Lisa, put her down for the evening with a pile of books, which, upon finishing, she flung out of the crib to the floor until she fell fast asleep. Her interest in science as a young girl was limited to the capture and subsequent study of fireflies in a jar, instead she was often seen roaming the property of the rectory in Catskill, New York with a pencil and pad, which she employed to create elaborate stories and drawings. How she ended up in science is anyone's guess. Perhaps it was because a Christmas-wish for a giant life-sized Barbie was replaced with a microscope- we may never know. Libby's childhood and stint in New York State ended in 1994, when her family moved to Laconia, New Hampshire, amongst the White Mountains and many lakes with exotic names, such as Winnipesaukee and Opechee. After an exemplary high school career at Tilton School, chosen based on the baroque appearance of its library, she attended Bowdoin College, an elite liberal arts institution nestled in the beautiful, progressive New England town of Brunswick, Maine. She selected Bowdoin because she liked the giant armchairs and sun design on the floor in the student union. At Bowdoin, Libby studied Biology and enjoyed genetics courses taught by Professor William Steinhart, who insisted that she call him Bill, and whom to

this day she still refers to as Professor Steinhart. Libby excelled in developmental biology, conducting independent research on Fetal Alcohol Syndrome with Missy Niblock, a visiting professor who hailed from North Carolina. With Missy's encouragement, Libby applied to the Department of Environmental and Molecular Toxicology at North Carolina State. In 2003, she entered the laboratory of Dr. Yoshiaki Tsuji to study the genetic regulation of ferritin H by oxidative stress. Under Yoshi's guidance, she learned the art of molecular biology and scientific method. She also regained her inclination for sporadic hysterical laughter. In a twist of fate, she was once again surrounded by men and fireflies. During this time, she met the first love of her life, Oliver McGuzzio, a giant, white Labrador retriever, at Wake County Animal Control on a warm sunny day in June 2004, and the second, Tom Hubscher, at the Millbrook Exchange Dog Park on November 5, 2005. In the future, she will undoubtedly return to her daydreaming and writing as she continues in her scientific endeavors. After completion of her doctorate, Libby will be working as a scientific writer/editor at Educational Training Systems International, and hopes to write and publish books. She and Tom continue to reside in Apex, NC with their four darling cats: Alois, Bixby, Jasmine, and Keyser Soze, and their dogs, Ollie and Kali.

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Rotenone Induces Transcription of the Ferritin H Gene by Oxidative Stress-Mediated Activation of an Antioxidant Responsive Element

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ABSTRACT

Tight regulation of intracellular iron levels in response to mitochondrial dysfunction is an important mechanism that prevents oxidative stress, thereby limiting cellular damage. Here, we describe a cytoprotective response involving transcriptional activation of ferritin H in response to the mitochondrial complex I inhibitor and neurotoxic compound, rotenone. Rotenone exposure increased ferritin H mRNA and protein synthesis as shown by Northern blotting and in vivo ^{35}S-methionine labeling and immunoprecipitation experiments. Transient transfection of ferritin H promoter luciferase reporter into NIH3T3 cells showed that ferritin H is transcriptionally activated by rotenone through an antioxidant responsive element (ARE). Chromatin immunoprecipitation and gel retardation assays showed that rotenone treatment enhanced binding of Nrf2 and JunD transcription factors to the ARE. In addition, rotenone induced production of reactive oxygen species (ROS), and pretreatment with N-acetylcysteine abrogated ferritin H mRNA induction by rotenone, suggesting that this response is mediated by oxidative stress. Furthermore, reduced ferritin H expression by siRNA enhanced ROS production and sensitized cells to rotenone-induced apoptosis. Taken together, these results suggest that ferritin H transcription is activated by rotenone via an oxidative stress-mediated pathway leading to ARE activation, and may be critically important to protect cells from mitochondrial dysfunction and oxidative stress.

INTRODUCTION

Iron is a vital element mandatory for metabolic processes and the function of many enzymes, including cytochrome P450s and ribonucleotide reductase (1); however, a surplus of free iron leads to the formation of reactive oxygen species (ROS) via the Fenton reaction, and is potentially harmful to the cells (2). Thus, cells have an intricate system of control of intracellular iron levels. Ferritin is a nanocage protein that functions to sequester free intracellular iron that may become toxic to the cells (3). In vertebrates, there are 2 ferritin subunits, heavy and light, which coassemble in 24 subunit bundles to form a channel that encloses iron (4). The heavy, or H, subunit is catalytically active, inducing oxidation of ferrous iron (Fe(II)) to ferric iron (Fe(III)), and causing aggregation of the oxidized iron inside the core, while the light (L) subunit does not have ferroxidase activity and may serve a structural function (5,6). In this manner, ferritin H functions to protect cells against iron mediated oxidative stress (7,8). Because of its important role, ferritin is tightly regulated at both the transcriptional and translational levels. Levels of ferritin protein are controlled by a well-characterized translational repression system in response to free iron levels in the cells (9). The 5' untranslated region of ferritin message contains an iron responsive element, to which the iron regulatory proteins, IRP1 and IRP2, bind in low iron concentrations, thereby blocking translation when necessary (10,11). In addition to iron, ferritin levels are also altered by other stimuli. TNFα was shown to stimulate ferritin H expression, but not ferritin L (12), through transcriptional activation via an upstream promoter region containing an NFkB site (13-15). Our subsequent studies revealed an antioxidant responsive element (ARE) in the far upstream region of the promoter that is necessary for the transcriptional activation of ferritin in response to various oxidative stressors, including H_2O_2, tBHQ, and

Hemin (16-18). A similar ARE element was also identified in ferritin L gene (19). The ARE is a highly conserved enhancer element in various phase II genes involved in detoxification or with antioxidant properties (20,21). It allows for the activation of a battery of antioxidant genes, including glutathione-s-transferases, NADH quinone oxidoreductase 1 and heme oxygenase 1 (20,22-25), under chemical and oxidative stress conditions. The ferritin H ARE contains a bidirectional AP1-like and AP-1/NFE2 sequence, to which basic leucine zipper (bZip) transcription factors including JunD and NFE2-related factor 2 (Nrf2) bind (16,18,26). This facilitates activation of ferritin H transcription in conditions of oxidative stress. Our recent studies demonstrated that JunD was phosphorylated following oxidative stress, and subsequently its binding to the ARE increased (18).

A large body of evidence indicates a correlation between increased oxidative stress and Parkinson's disease (PD) incidence (27,28). Recent studies have shown that iron chelators can block 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrapyridine (MPTP) induced dopaminergic cell death (29,30). Excess free iron may be the catalyst in the production of deleterious ROS that are responsible for damage to DNA, lipids, and proteins, and may ultimately lead to the deletion of dopaminergic neurons (31). Furthermore, the insults that induce neuronal degeneration are intimately connected with oxidative stress (32). Rotenone, a mitochondrial complex I inhibitor, induces oxidative stress; however, despite its universal effect, it leads to the specific deletion of dopaminergic neurons (33,34), suggesting that they are highly sensitive to oxidative stress.

We hypothesized that rotenone would evoke cellular defense mechanisms through increased expression of ferritin H via an oxidative stress pathway. In our present study we show that

ferritin H expression is increased at the transcriptional level following rotenone exposure. We further characterized that rotenone specifically activates the ferritin H ARE, and increases the binding of the oxidative stress responsive bZip transcription factors, JunD and Nrf2, to it. Furthermore, rotenone-mediated transcriptional activation of the ferritin H gene is oxidative stress dependent. Finally, knocking down ferritin H expression by siRNA propagated the generation of ROS and sensitized cells to rotenone-mediated apoptosis, suggesting that rotenone induced transcriptional activation of the ferritin H gene via the ARE is cytoprotective.

EXPERIMENTAL PROCEDURES

Cell culture.

NIH3T3 mouse fibroblasts were obtained from American Type Culture Collection. NIH3T3 cells were cultured at 37°C and 5% CO_2 in a humidified atmosphere in Dulbecco's Modified Eagle's Medium containing 1mM sodium pyruvate, 4 mM L-glutamine, and 4.5g/L glucose with 10% bovine calf serum (Hyclone), and penicillin/ streptomycin. Rotenone and tert-butylhydroquinone (tBHQ) were purchased from Sigma and were dissolved in DMSO. H_2O_2 and N-acetylcysteine (NAC) were purchased from Calbiochem. H_2O_2 was diluted in sterile phosphate-buffered saline (PBS), and NAC was reconstituted in media and adjusted to pH 7.4 with NaOH.

Northern Blotting.

Total RNA from NIH3T3 cells was isolated using TRIzol (Invitrogen). 5-10 ug RNA was separated on a 1.1% agarose, formaldehyde gel, and separated RNA was transferred to a 0.45 micron nitrocellulose Protran BA85 membrane (Whatman, Schleicher & Schuell) by capillary transfer. Northern blotting was performed using an α-^{32}P-dCTP random primer labeled 0.9 kb fragment of ferritin H human cDNA probe.

Plasmids and DNA transfections.

pGL3–0.22 kb mouse ferritin H luciferase (mFH-Luc) was constructed by digesting pGL3–4.8 kb mFH-Luc with SmaI to remove the upstream 4.6 kb and then self ligating the remaining vector and 0.22 kb promoter sequence. ARE, and double mutant ARE-Luc plasmids were obtained by blunt end ligation of the oligonucleotides into the –0.22 kb mFH-

Luc plasmid (below) following oligonucleotide purification by urea denaturing polyacrylamide gel electrophoresis, Sephadex G-25 column purification, and subsequent annealing. The sequences are:

wt ARE SENSE:

CATGACAAAGCACTTTTGGAGCCCAACCCCTCCAAAGGAGCAGAATGCTGAGTC
ACGG

wt ARE ANTISENSE:

CCGTGACTCAGCATTTCTGCTCCTTTGGAGGGGTTGGGCTCCAAAAGTGCTTTGT
CATG

mARE SENSE:

CA**C**A**A**CAAAGCACTTTTGGAGCCCAACCCCTCCAAAGGAGCAGAA**C**A**A**CTGAGTC
ACGG

mARE ANTISENSE:

CCGTGACTCTAGTGTTCTCTCCTTTGGAGGGGTTGGGCTCCAAAAGTGCTTTGTT
GTG

NIH3T3 cells were transfected with 5ug of the indicated plasmid DNA via electroporation with a BioRad GenePulser XL using NIH3T3 preset conditions, $5x10^6$ cells/0.2cm cuvette. Cells were then plated into 35mm dishes (10 dishes) with $5x10^5$ cells, 0.5ug DNA per dish. Following a 24-hour recovery period after electroporation, cells were treated as indicated for

24 hours, and preparation of cell lysates and luminometry were performed with luciferase assay kit (Promega).

Gel retardation Assay.

Nuclear extract preparation, binding reactions, and electrophoretic mobility shifts were previously described (35). JunD (sc-74X), JunB (sc-46X), and cJun (sc-45X) antibodies were obtained from Santa Cruz Biotechnology, Inc.

ChIP Assay.

Chromatin immunoprecipitation (ChIP) assays were performed according to a minor modification of Upstate Biology's ChIP assay protocol as previously described (16). A total of $1x10^6$ cells/100-mm plate were treated with 1uM rotenone for 4 hours, followed by formaldehyde cross-linking of chromatin and preparation of lysates. (Upstate Biology). 2 ul of the following antibodies were used for immunoprecipitation: rabbit IgG (alpha diagnostics), anti-Nrf2 (sc-722X), anti-JunD (sc-074X), (Santa Cruz Biotechnology). Quantitative PCR was performed in the presence of 1 uCi α-^{32}P dCTP/reaction, while using specific primers designed to amplify a 230 bp region within the mouse ferritin H promoter that contains the ARE:

Forward:

5'GGCCCCTCTGTTCTGTACAATACTAGCTC-3'

Reverse:

5'TAACCACAAAACCACAGCCCTCCAG-3'

PCR reactions were run on an 8% acrylamide gel and visualized by autoradiography.

Western blotting.

Total cell lysates were subjected to electrophoresis with a 15% or 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), followed by wet transfer of separated proteins to an Immobilon-P membrane (Millipore). Membranes were blocked for one hour in 5% skim milk in Tris buffered saline with 0.1% Tween 20, and subsequently incubated overnight at 4°C with anti-ferritin H antibody (Abcam 1:20,000). Recombinant human ferritin H and L were purchased from Calbiochem.

^{35}S-Translabeling/immunoprecipitation.

Exponentially growing NIH3T3 cells were treated with either rotenone or ferric ammonium citrate for the indicated times. Following treatment, media was removed and methionine/cysteine deficient DMEM containing 10% dialyzed bovine calf serum was added. Simultaneously, 10 uCi/ml of ^{35}S methionine/cysteine (GE Healthcare) was added to each dish. Cells were incubated under normal culture conditions for one hour. Total cell lysates were prepared, and pre-cleared with rabbit serum (CAPEL) and protein A agarose (Calbiochem) overnight. Total incorporation of ^{35}S was measured using TCA precipitation and scintillation counting. Input protein for immunoprecipitation was determined by adding equal counts ($1x10^6$ cpm) for each immunoprecipitation reaction. 6 ul of Anti-Ferritin antibody (DAKO, A133) and 20 ul of protein A agarose were utilized for overnight immunoprecipitation at 4°C. Finally, the resulting immunoprecipitates were subjected to SDS-PAGE as described above, the gel was dried, and exposed to film for 4-10 days at –86° C.

SiRNA transfection.

Ferritin H siRNA was purchased from DHARMACON, Inc, siGENOME duplex D-045965-01 mouse FTH, NM_010239.

Sense:5'CAAGAAUGAUCCCCACUUAUU-3'

Antisense: 5'PUAAGUGGGAUCAUCAUUCUUGUU-3'

Briefly, $1x10^5$ cells/35 mm dish were plated and cultured under normal conditions in serum-containing, antibiotic-free media (2 ml) until cells reached approximately 80% confluency. 16 ul DharmaFECT3 (Dharmacon) in a final volume of 200 ul OPTI-MEM media (Invitrogen) was added to 7.5ul of 20 uM (150 pMol) siRNA dissolved in 200 ul of OPTIMEM, and the mix was incubated for 20 minutes. After formation of RNA complexes, the 400 ul mix was added to dishes containing 1.6 ml of fresh, antibiotic-free DMEM with 10% bovine calf serum, and the cells were cultured under normal conditions. After 24 hours, cells were split into a 6-well plate for treatment. Whole cell extracts were obtained, and subjected to Western blotting as described above to examine ferritin H protein expression. In other cases, cells were trypsinized and assessed for ROS production and apoptosis using flow cytometry.

Detection of ROS. NIH3T3 cells in 35mm dishes were incubated with 5 uM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Molecular Probes) dissolved in molecular biology grade DMSO (Calbiochem) in phenol red free DMEM containing 10% bovine calf serum for 30 minutes. This cell permeable probe is non-fluorescent until the acetate group is removed by intracellular esterases, and the deacetylated species is subsequently oxidized by intracellular ROS. After loading, the

unincorporated dye was washed out with phenol red-free complete medium, and rotenone (5 uM) and H_2O_2 (50 uM) were added for 0.5 hours. Following treatment, cells were trypsinized and resuspended in phenol red complete medium, and cell pellets were collected after centrifugation at 2000 rpm for 3 minutes. The resultant pellets were suspended in 500 ul phenol red free complete medium for flow cytometry on a Becton-Dickinson FACSCalibur.

Detection of Apoptosis. To detect apoptosis, Annexin V conjugated with AlexaFluor647 (Molecular Probes-Invitrogen) was utilized. Annexin V detects the presence of phosphatidylserine on the outside of apoptotic cells. NIH3T3 cells in 35 mm dishes were treated with 12.5 or 25 uM rotenone for 8 hours. Cells were trypsinized, and the resulting cell pellets were stained according to the manufacturer's protocol, with the following modification: 2.5 ul Annexin v AlexaFluor 647 (Molecular probes) was added to the 100 ul cell suspension. AlexaFluor 647 fluorescence (indicating Annexin V positive cells) was assessed via flow cytometry on a Becton-Dickinson FACSCalibur.

RESULTS

Rotenone induces ferritin H- To investigate the effect of the mitochondrial complex I inhibitor, rotenone, on ferritin H expression, we examined the levels of ferritin H mRNA following rotenone exposure by Northern blotting. As shown in Fig. 1, treatment of NIH3T3 cells with increasing concentrations of rotenone for 24 hours increased ferritin H message in a dose dependent manner. The inductive effect of the highest dose used was similar to that of tBHQ, an activator of ferritin transcription (17,36). Our previous studies indicated that H_2O_2 inhibits ferritin translation within 1-2 hours due to transient IRP activation (17); therefore, we next investigated whether ferritin H protein synthesis was transiently repressed following rotenone treatment. We exposed NIH3T3 cells to 1 uM rotenone for various times over a period of 24 hours, and subsequently subjected them to ^{35}S-methionine/cysteine translabeling. As shown in Fig. 2, increased ferritin protein was observed 1 hour following treatment and was maintained for 24 hours. This suggests that IRP (translational repression system) is not activated by rotenone treatment, since there was no transient reduction in the level of ferritin H protein synthesized at any time point following the initiation of treatment. In addition, ferritin protein exhibited a dose responsive increase following 24-hour treatment with 0.1, 0.5, and 1 uM rotenone (Fig. 2b).

Ferritin H is transcriptionally activated by rotenone through the ARE- Since we had observed increased levels of mRNA and protein synthesis following rotenone treatment, we were interested in whether or not ferritin H was transcriptionally activated. To test this, we transiently transfected NIH3T3 cells with a firefly luciferase reporter fused to 4.8 kb or 0.22 kb of the 5' region of the mouse ferritin H promoter, and treated them for 24 hours with

rotenone. Increasing concentrations of rotenone specifically activated the -4.8 kb promoter in a dose-dependent manner, but had no effect on the -0.22 kb promoter (Fig. 3). This suggests that the increase in ferritin H mRNA may be result of transcriptional activation.

The -4.8 kb region of the mouse ferritin H promoter that was activated by rotenone contains an ARE, located 4.1 kb upstream of the transcription start site (37). Next, we asked whether or not the ARE was involved in transcriptional activation of ferritin H by rotenone. We cloned the wt ARE or double mt ARE, in which both the AP-1 like and AP-1/NFE2 sites contain critical mutations that abrogate transcription factor binding, into the minimum 0.22 kb promoter reporter, and employed them for transient transfection assays in NIH3T3 cells. Fig. 3b shows that insertion of the wt ARE was sufficient for promoter activation by rotenone treatment, while introduction of mutations in the AP-1 like and AP-1/NFE2 sites abrogated rotenone mediated promoter activation. This indicates that not only is the ARE activated by rotenone treatment, but that the AP-1 binding sequences are also critical for rotenone mediated transcriptional activation of the ferritin H gene.

Nrf2 and JunD binding to the ferritin H ARE is enhanced by rotenone treatment- Given that the mutations of the AP-1 like and AP-1/NFE2 sites blocked ferritin H transcriptional activation in our transient transfection assays, we investigated whether transcription factor binding to the ARE was altered following rotenone treatment. First, gel retardation assays demonstrate that total protein binding to the ARE was increased by rotenone treatment in NIH3T3 cells (Fig. 4a). To assess the role of Nrf2 (a major transcription factor responsible for regulation of the ARE in various phase II genes) (38) and JunD, we performed ferritin H

ARE ChIP assay. As shown in Fig. 4b, in vivo binding of Nrf2 and JunD to the ARE is increased following rotenone treatment, suggesting that they are involved in the transcriptional activation of the ferritin H gene in response to rotenone treatment.

ROS production is involved in rotenone-mediated ferritin H induction- Given the results of rotenone-mediated ferritin H ARE activation in this study, we then hypothesized that rotenone induces ferritin H through in an oxidative stress dependent manner. To test this hypothesis, we assessed rotenone's propensity to produce ROS, using the dye CM-H_2DCFDA, which is taken up by cells and is non-fluorescent in its acetylated, reduced form. Once localized in the cell, intracellular esterases deacetylate the dye, allowing for its oxidation by ROS. The oxidized dye exhibits a shift in its emission spectra to the fluorescein range. Treatment of NIH3T3 cells with 5 uM rotenone for 0.5 hours resulted in a significant increase in the percent fluorescent positive cells, indicating that rotenone induces production of ROS and has the potential to cause oxidative stress (Fig. 5a). To reveal the role of oxidative stress in the induction of ferritin H by rotenone, we assessed the ferritin H mRNA levels of NIH3T3 cells treated with vehicle, rotenone, or rotenone following pre-administration of N-acetylcysteine (NAC), which is known to prevent production of reactive oxygen species by raising levels of glutathione. Indeed, NAC pretreatment abrogated the increase in ferritin H mRNA by rotenone treatment, but NAC treatment alone had negligible effects on ferritin H mRNA levels (Fig. 5b). Taken together, this indicates that rotenone activates ferritin H transcription by an oxidative stress-dependent mechanism, potentially involving increased JunD and Nrf2 binding to the ARE.

Ferritin H knockdown increases ROS production and sensitizes NIH3T3 cells to rotenone-induced apoptosis- Our observation that oxidative stress was required for the induction of ferritin H by rotenone, led us to assess the role of ferritin H in protecting cells from rotenone induced oxidative stress and apoptosis. To determine whether ferritin H is cytoprotective against rotenone mediated oxidative damage, we utilized ferritin H siRNA to transiently decrease ferritin expression. Ferritin H siRNA (FH) decreased the expression of ferritin H protein in NIH3T3 cells by 50% compared to non-targeting siRNA (NT) (Fig 6a, top). Following either NT or FH siRNA transfection, cells were treated with increasing concentrations of rotenone and examined for ROS production. As shown in Fig. 6a, FH siRNA NIH3T3 transfectants had increased ROS production following rotenone treatment compared to NT siRNA transfectants. We then asked if ferritin H knockdown cells are more susceptible to cytotoxicity induced by rotenone. To this end, rotenone-induced apoptosis was detected using fluorescently labeled annexin V, a protein that binds externalized phosphatidylserines. FH siRNA transfected cells had higher levels of annexin V staining, indicating that a greater percentage of the ferritin H knockdown cells were undergoing apoptosis (Fig. 6b). Taken together, these results suggest that decreased ferritin H expression increases ROS production and sensitizes cells to rotenone, and that a deficient ferritin H response to rotenone-induced oxidative stress causes cell death.

DISCUSSION

Maintenance of iron homeostasis is critically important in preventing oxidative cell damage via the Fenton reaction. Ferritin is a major protein involved in the regulation of intracellular free iron levels. It sequesters iron within its shell, thereby blocking iron from participating in reactions that generate free radicals. In some instances where cellular oxidative load is increased, cells may be more susceptible to iron mediated production of ROS and damage. We, along with others, demonstrated that ferritin is upregulated in response to a battery of different oxidative stressors (16,17,19,26). In mouse erythroleukemia cells, overexpression of ferritin H reduced free iron and decreased production of ROS post-treatment with H_2O_2 (39,40). HeLa cells overexpressing ferritin H were deficient in labile iron and resistant to oxidative stress (41). Deficiencies in ferritin lead to cellular profiles of oxidative stress and iron accumulation (7).

Oxidative stress and iron also have both been implicated in the pathogenesis of Parkinson's disease. Increased iron concentrations and oxidative damage have been observed in damaged regions of the *substantia nigra* in both human cases of Parkinson's disease (PD) and in animal models (42-44). Taken together with rotenone-induced experimental models of Parkinson's disease (33), we hypothesized that rotenone may increase levels of ferritin H. In fact, we observed that ferritin H is induced following rotenone exposure at the mRNA and protein synthesis levels (Figs. 1, 2).

Our previous studies demonstrated that H_2O_2 produces a transient activation of IRP binding to the IRE, thus conferring a temporary reduction in ferritin H synthesis (17). Our results in

this study demonstrate that ferritin H synthesis was not reduced at any time point following rotenone exposure, suggesting that IRP was not activated by rotenone. Like rotenone, tBHQ also does not activate IRP (unpublished data). This may potentially be due to the differences in the ROS produced by each stressor. tBHQ and rotenone produce ROS indirectly by redox cycling and leakage of electrons from the electron transport chain, respectively (45). H_2O_2, on the other hand, is a ROS of its own right, and may directly interact with IRP, leading to its activation.

We were interested in the mechanism of ferritin increase by rotenone. Upregulation of ferritin following chronic MPTP administration in mice was demonstrated by cDNA microarray (46), but little is known about the mechanism of transcriptional activation of ferritin, or the signaling pathway responsible for the inductive effect of neurotoxicants. We showed that ferritin H is transcriptionally activated by rotenone in a dose dependent manner (Fig. 3). Furthermore, this activation was mediated through the ARE. Wild type ARE insertion alone was sufficient to produce activation similar to that observed by the 4.8kb promoter. In addition, mutation of the AP-1 like and AP-1/NFE2 binding sites abrogated the activation by rotenone (Fig. 3), suggesting the importance of these sites in the activation of ferritin H transcription. Indeed, as shown by our gel retardation assay, enhanced total protein binding to the ARE was observed following rotenone treatment, in which increased Nrf2 and JunD binding to the ARE was detected by ChIP assay (Fig. 4). This lead us to propose that oxidative stress is downstream of the complex I inhibition by rotenone in the cascade of events that lead to ferritin H transcriptional activation. Confirming our hypothesis, addition of the glutathione precursor, NAC, blocked rotenone-mediated ferritin H mRNA induction

(Fig *5*), suggesting that oxidative stress is a necessary cue in ferritin H activation by rotenone. Recently, two studies demonstrated a protective function of Nrf2 in mitochondrial dysfunction and oxidative stress caused by the complex II inhibitor, 3-nitropropionic acid (47,48). Interestingly, tBHQ has been shown to stabilize Nrf2 and enhance Nrf2-mediated ARE activation. Furthermore, tBHQ conferred cytoprotection against 6-hydroxydopamine induced oxidative stress in neuronal cells (49). These findings, along with our results in this study suggest that ferritin H may be a target gene in the response to mitochondrial dysfunction and oxidative stress produced by rotenone.

To investigate the cytoprotective role of ferritin against rotenone toxicity, we examined the effect of decreased ferritin H expression on cells' sensitivity to rotenone by siRNA transfection. FH siRNA transfection caused increased production of ROS and apoptosis in response to rotenone treatment compared to NT siRNA transfected cells (Fig. 6a). We also observed that knocking down ferritin H expression resulted in increased ferritin L expression in NIH3T3 cells (Fig. 6a). This is consistent with a recent study reported by Cozzi et al., showing that ferritin H knockdown increased ferritin L expression in HeLa cells, and that increased ferritin L expression did not affect iron availability (7). These results suggest that alterations in ferritin H expression may directly contribute to the altered iron metabolism and increased oxidative stress present during the disease progression of PD. In fact, it was reported that overexpression of ferritin H prior to MPTP exposure in a mouse model of PD conferred resistance to the neurotoxic insult (30).

Interestingly, in brain tissue from individuals afflicted with PD, the Fe(II)/Fe(III) ratio is 1:2, compared to 2:1 in normal *substantia nigra pars compacta* (50). This indicates that an increased amount of Fenton reactions occur during the progression of PD. Also, regions of iron accumulation colocalize with those of neuronal death (51), and iron chelators prevent alpha synuclein translocation and mitochondrial aggregation, two hallmark events in the pathogenesis of PD (52). It is also interesting to note that increased levels of a number of antioxidant enzymes such as glutathione peroxidase and catalase are evident in the PD tissues (43,53), while there are also increases in byproducts of lipid peroxidation in PD affected structures (54).

In addition to ferritin, a number of other metal regulatory/antioxidant genes have been implicated in PD. For instance, metallothionein overexpression was shown to be cytoprotective against neurotoxic insult (55). Like ferritin H, metallothionein also contains an ARE and is regulated by both oxidative stress and iron (56). It seems likely that a common ARE mediated mechanism of phase II gene activation is a critical cytoprotective response to neurotoxicants. Ferritin H as a major iron sequestering protein may be an important component among the battery of activated genes. Our results in this study may shed light on the potential role of ferritin regulation in the pathogenesis of PD, as well as providing information as a potential target for chemoprevention.

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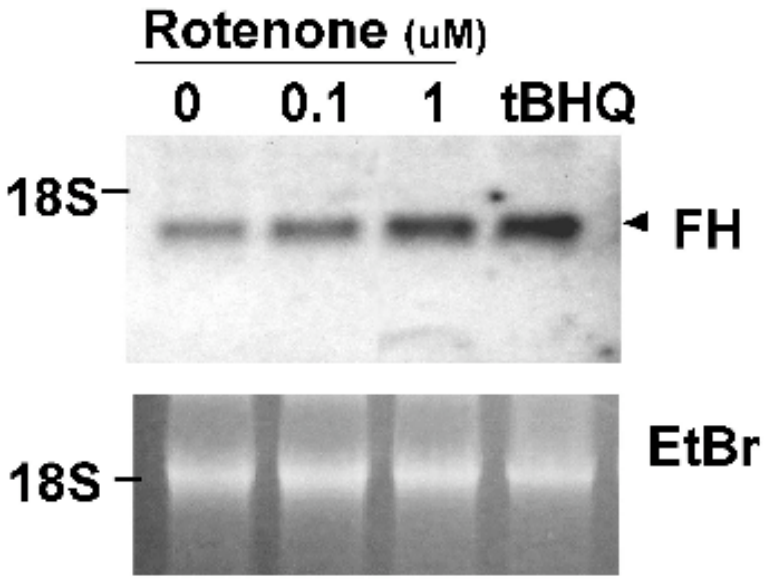

Fig. 1 Rotenone induces ferritin H mRNA. NIH3T3 cells were treated with either 0, 0.1, or 1 uM rotenone or 10 uM tBHQ for 24 hours. To assess equal loading and integrity of total RNA, ethidium bromide staining is shown below. The position of 18s ribosomal RNA is also indicated. A representative Northern blot result of 4 independent experiments is shown.

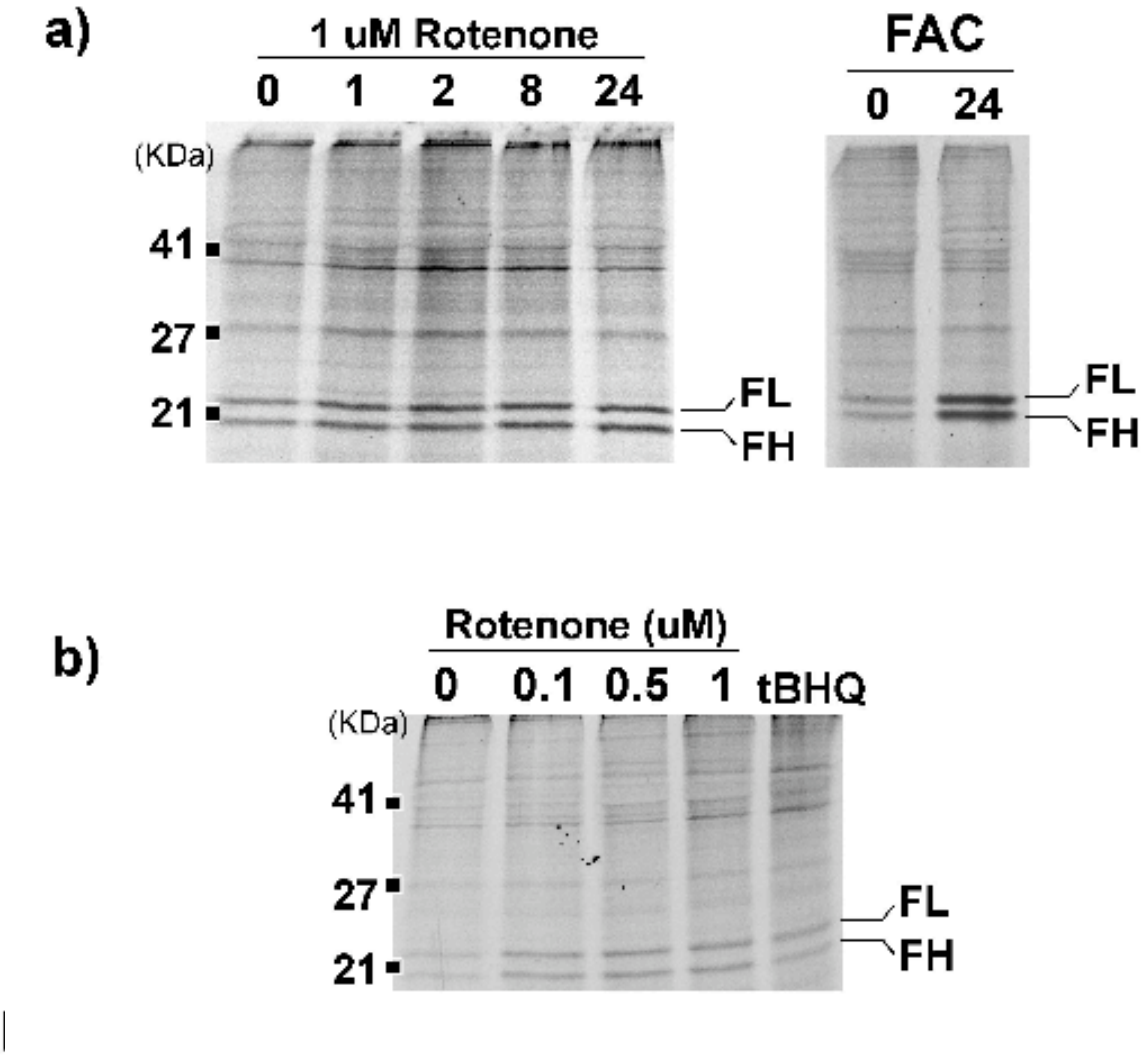

Fig. 2 Rotenone treatment results in increased ferritin H protein synthesis. a) NIH3T3 cells were incubated with 1uM rotenone for 0, 1, 2, 8, and 24 hours, and subsequently subjected to in vivo labeling of newly translated protein with ^{35}S-methionine/cysteine. 1x10^6 TCA insoluble counts were subjected to immunoprecipitation with anti-ferritin antibody and subsequently separated by SDS-PAGE. NIH3T3 cells were treated with 5ug/ml ferric ammonium citrate (FAC) as a positive control for ferritin protein induction and ^{35}S-labeled at 0 and 24 hours. The resulting ferritin H (FH) and ferritin L (FL) bands are indicated by arrows. **b)** NIH3T3 cells were treated with 0, 0.1, 0.5, and 1 uM rotenone or 10 uM tBHQ for 24 hours and subjected to in vivo ^{35}S-methionine/cysteine labeling, immunoprecipitation and SDS-PAGE as described above. Representative images from 3 independent experiments are shown for each.

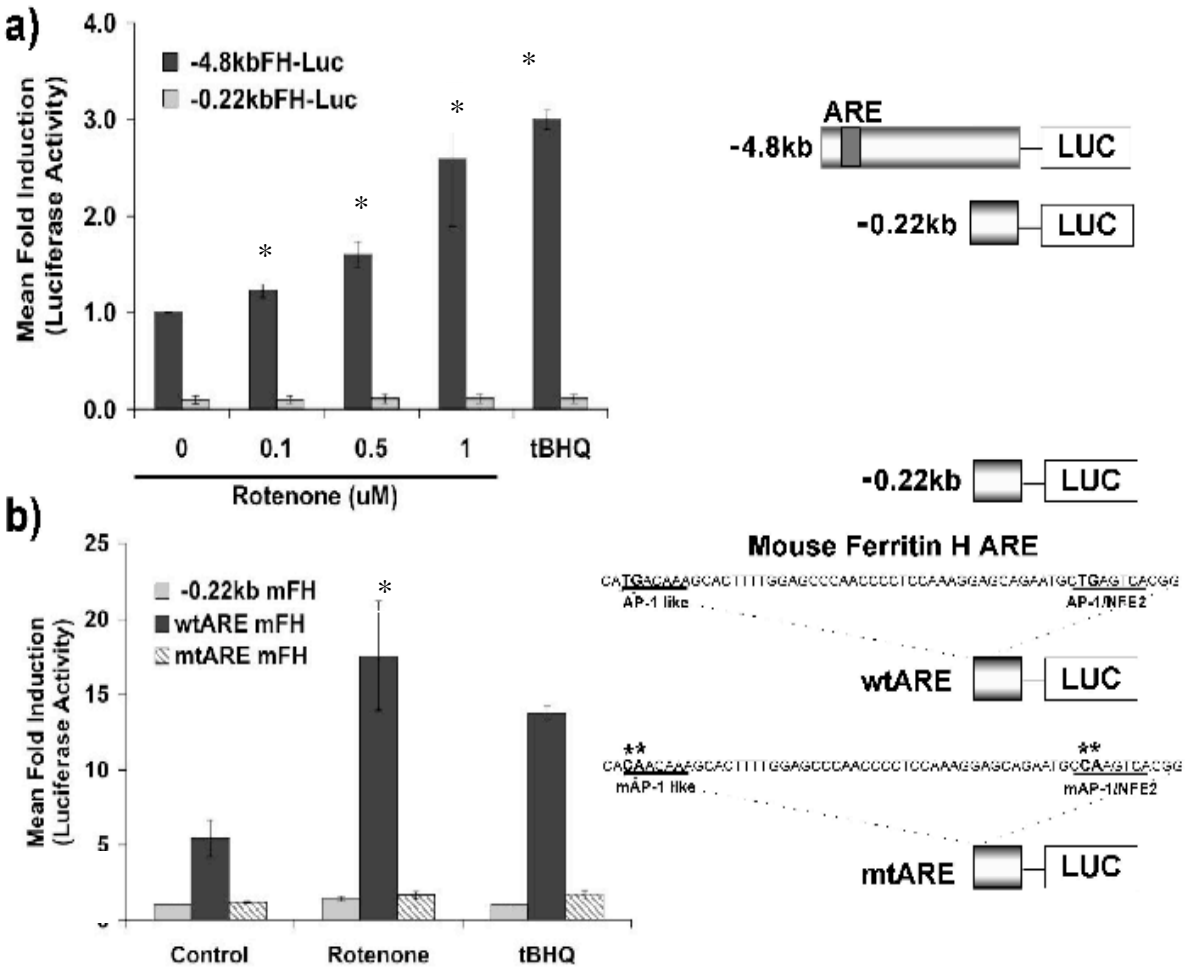

Fig. 3 Transcriptional activation of ferritin H by rotenone. a) NIH3T3 cells were transfected via electroporation with 0.5 ug of either -0.22 kb FH or -4.8 kb FH firefly luciferase promoter reporter constructs. After a 24 hour recovery period, cells were treated with 0, 0.1, 0.5, or 1.0 uM rotenone, or 10 μM tBHQ and incubated for 24 hours. Lysates were collected and assessed for transcriptional activation via luminometry. –4.8 kb FH, 0 uM rotenone treatment was set to 1 to calculate mean fold induction. Standard errors of means (S.E.M.) are shown, where n=5 independent experiments. **b)** NIH3T3 cells were transfected as above with -0.22 kb FH, wt ARE insertion, or mt ARE insertion fused to a firefly luciferase reporter construct. Cells were treated in duplicate for 24 hours with either 1.0 μM rotenone or 10 μM tBHQ. Samples were normalized for recovery and transfection efficiency differences using a co-transfected internal control pRL-EF (elongation factor-renilla luciferase plasmid). The -0.22 kb FH-Luc control value was set to 1 to calculate mean fold induction. S.E.M. shown, n=4 independent experiments. A * denotes statistical significance compared to 0 uM rotenone (**a**) or control (no treatment) (**b**), defined as $p<0.001$, as determined by Student's t-test.

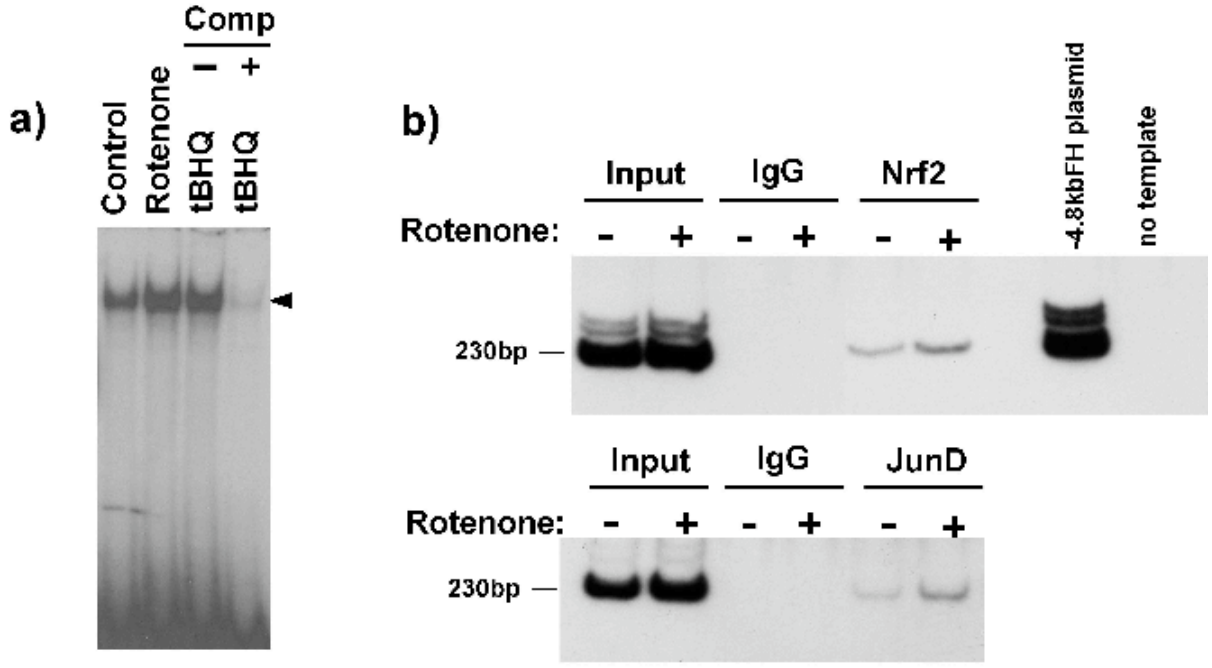

Fig. 4 Rotenone alters transcription factor binding to the ARE. a) 50 ug of nuclear extracts from NIH3T3 cells treated with 1uM rotenone or 10 uM tBHQ for 4 hours, or untreated cells (control) were subjected to gel retardation assay using a probe for the AP-1/NFE2 site in the ferritin H ARE. Addition of 50x excess cold probe competitor to lane 4 is indicated by +. **b)** NIH3T3 cells treated with 0 or 1 uM rotenone for 4 hours were used for ferritin H ARE ChIP assay. Primers specific to a region of the mouse ferritin H promoter that contains the ARE were employed for PCR with the input DNA, or the DNA obtained following immunoprecipitation with either rabbit IgG, Nrf2 specific antibody, or JunD specific antibody. The resulting 230 bp PCR product is shown. –4.8 kb FH indicates the use of mouse 4.8 kb FH-Luc plasmid DNA as template for a positive control, and NC is no template. Representative images are shown of 3 independent experiments for (**a**) and (**b**).

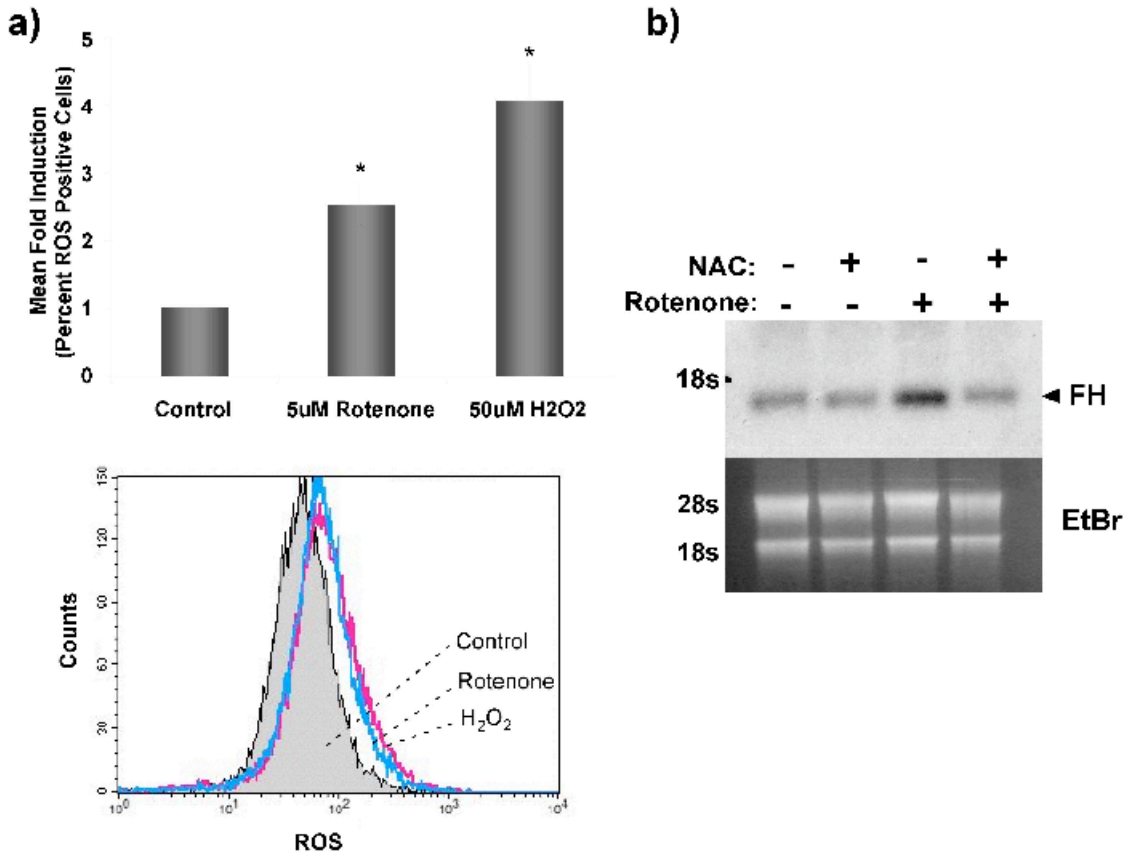

Fig. 5 ROS production is involved in rotenone-mediated ferritin H mRNA induction. **a)** NIH3T3 cells were pre-loaded with the ROS sensitive dye, CM-H_2DCFDA, for 0.5 hours, and then treated with 5 uM rotenone or 50 uM H_2O_2 or left untreated (Control) for 0.5 hours. Levels of ROS, as indicated by FITC fluorescence, were assessed via flow cytometry. Mean fold induction of the number of ROS positive cells was calculated by setting the control levels to 1. S.E.M. are shown, where n=3 independent experiments. A * denotes statistical significance compared to control, defined as $p<0.001$ by Student's t-test. A representative histogram for ROS fluorescence from 3 independent experiments, showing number of counts vs. relative FITC fluorescence, is shown at the right. **b)** NIH3T3 cells were treated with 0 or 1 uM rotenone for 24 hours (indicated by – or + respectively, lower row, marked rotenone), following a 2 hour pre-treatment with 0 or 10 mM NAC (indicated by – or + respectively, upper row, marked NAC). Resulting total RNA was subjected to Northern blotting with a ferritin H cDNA probe. EtBr staining is shown below for loading and RNA integrity. The positions of 18s and 28s RNA bands are indicated. A representative image of 3 independent experiments is shown.

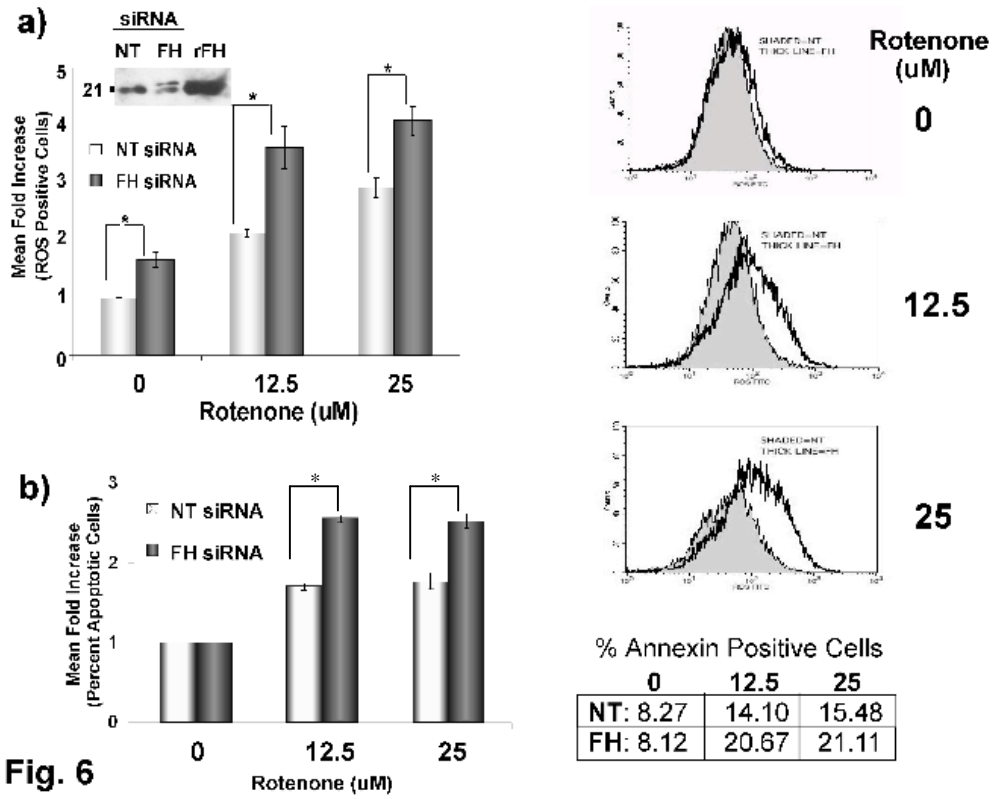

Fig. 6

Fig. 6 Ferritin H knockdown increases ROS production and sensitizes NIH3T3 cells to rotenone-induced apoptosis. a) NIH3T3 cells were transfected with non-targeted (NT) siRNA or ferritin H (FH) siRNA. Resulting ferritin H protein levels were assessed by Western blotting with anti-ferritin antibody. Recombinant human ferritin H (rFH) was included as a Western blotting control. A representative Western blot is shown. NT and FH siRNA transfectants were also treated with 0, 12.5, and 25 uM rotenone for 0.5 hours following loading for 0.5 h with CM-H_2DCFDA and ROS levels were analyzed by flow cytometry. Resulting histograms showing counts (y-axis) vs. ROS FITC fluorescence (x-axis) are shown at right. The shaded area represents the histogram of NT siRNA transfectants, and the thick black line is the FH siRNA transfectants. The mean fold increase in ROS positive cells NT and FH siRNA transfectants is summarized at left. The difference between NT and FH siRNA transfectants treated with 0 uM rotenone was set to 1. Standard error is shown for n=3 independent experiments. **b)** NT and FH siRNA transfectants were treated with 0, 12.5, or 25 uM rotenone for 8 hours, and apoptosis was quantified using flow cytometry to measure the fluorescence of AlexaFluor 647 conjugated to Annexin V. Standard error is shown for n=3 independent experiments. Average percent annexin positive cells are summarized in table, at right. A * denotes statistical significance $p<0.005$, as determined by Student's t-test for both (**a**) and (**b**).

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Elevated Intracellular Calcium Enhances Ferritin H Expression Through an NFAT-Independent Mechanism

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ABSTRACT

Upon stimulation by antigen, a rise in intracellular Ca^{2+} is one of the initiating events in T-cell activation. A calcium-mediated signaling cascade that involves activation of calcineurin and the dephosphorylation and translocation of Nuclear Factor of Activated T-cells (NFAT) is triggered, and results in the transcriptional activation of gene targets, including IL-2. During this process, oxidative stress is produced. In the present study, we report that increased intracellular calcium leads to induction of the antioxidant protein ferritin H through an NFAT-independent pathway. Treatment of Jurkat T-cells with the calcium ionophore, ionomycin, led to increased ferritin H mRNA, and enhanced ferritin protein expression. Though NFAT translocated to the nucleus and bound and activated a consensus NFAT binding sequence located in the IL-2 promoter following ionomycin treatment, it did not activate ferritin H transcription despite a putative NFAT consensus sequence in the ferritin H antioxidant responsive element. In addition, cyclosporin A treatment did not abrogate the ionomycin-mediated increase in ferritin H mRNA. Analysis of mRNA stability following actinomycin D treatment revealed that ionomycin prolongs ferritin H mRNA half-life. Taken together, these results suggest that ionomycin-mediated induction of ferritin H may occur through posttranscriptional stabilization of mRNA.

INTRODUCTION

An elevation in intracellular calcium incites a signaling cascade, leading to the activation of T-cells in the immune response [1]. Increased calcium is responsible for the activation of calcineurin, a calcium-calmodulin dependent phophatase that is involved in transcriptional regulation of cytokine genes [2]. Studies have shown that calcineurin is integrally involved in diverse signaling pathways, which correlate with those controlled by calcium concentration, especially pathways that dictate lymphocyte function [3]. Calcineurin's major effector protein, NFAT, was originally described for its role in T-cell activation [4]. Classically, NFAT binds a highly conserved promoter sequence originally found in the upstream region of IL-2 gene; however, recently it has been shown to bind many other promoter targets, including a number of other interleukins, GM-CSF, IFN-gamma, TNF-alpha, and e-selectin [5].

During T-cell activation, the population of T-cells increases and reactive oxygen species are produced; however, following these events a large proportion of the population dies via an apoptotic pathway [6]. Calcium may lead directly to activation of the apoptotic pathway [7]. Recently, some evidence has shown that calcium may be released from the ER or taken up through channels during generalized stress [8-10]. It is thought that this calcium increase in the cell may be the compulsory signal for the cell to die [11]. It has also been proposed that ROS produced during T-cell activation may sensitize T-cells to undergo apoptosis [12].

ROS are potentially damaging to the cell, but because they are ubiquitous, cells have evolved antioxidant systems to convert them into more benign molecules [13]. In response

to oxidative stress, phase II enzymes are activated and upregulated. At the transcriptional level, such phase II genes, including glutathione-s-transferase, NADPH:quinone-oxioreductase-1, and heme-oxygenase-1, are induced via a conserved cis-element in their promoter, aptly named antioxidant responsive element (ARE) [14, 15]. Recently, our studies revealed an ARE in the 5' region of the human ferritin H promoter that is responsible for activation of transcription in response to such stimuli as hydrogen peroxide, tBHQ, and hemin [16, 17].

Ferritin H serves a cytoprotective role against iron-catalyzed formation of ROS under conditions of oxidative stress, where labile Fe^{2+} (Fe(II)) becomes an enabler of the production of the hydroxyl radical, which is capable of producing the most deleterious effects in the cell [18]. Ferritin is a nanobox protein designed to sequester free intracellular iron that may become toxic to the cell. In vertebrates, there are 2 subunits, heavy and light, which assemble in 24 subunit collections to create a channel that encloses the iron. The heavy, or H, subunit has ferroxidase activity, and is thereby able to interact with, and oxidize, Fe(II), causing it to aggregate inside the core [19]. Ferritin H functions to protect the cell against iron mediated oxidative stress [20, 21]. We, and others, have demonstrated that the transcriptional activation of ferritin H and other phase II genes through the ARE is mediated by AP-1 family member transcription factors, including JunD and Nrf2 [17, 22-25]. Ferritin translation, in contrast, is controlled by a well known mechanism involving the binding of an iron regulatory protein (IRP) to an iron responsive element (IRE) in the 3'UTR of ferritin mRNA [26]. Binding of IRP to IRE blocks translation, and occurs under conditions of free iron deficiency [27].

Very little is known about the responses that allow a cell to survive the concurrent intracellular calcium concentration and ROS production during T-cell activation, which may prevent apoptosis in selected cells in the population. Here, we show that the antioxidant protein, ferritin H is upregulated in response to elevated intracellular calcium. Enhanced ferritin H expression was independent of NFAT activity, and occurred through a transcription independent pathway. Interestingly, we identified an au-rich element, which is a putative binding site for a number of mRNA binding proteins involved in control of mRNA stability, in the 3' UTR of ferritin H mRNA, and increased calcium appeared to confer ferritin H mRNA stabilization. Taken together these results suggest that increased ferritin H expression after ionomycin treatment is mediated by the posttranscriptional stabilization of ferritin H mRNA. Furthermore, this upregulation may be part of a cytoprotective response to the concomitant ROS production or subsequent apoptotic signaling.

EXPERIMENTAL PROCEDURES

Cell culture: NIH3T3 and Jurkat E6-1 cells were purchased from American Type Culture Collection and cultured in Dulbecco's Modified Eagle's Medium with 10% Bovine Calf Serum and RPMI with 10% Fetal Bovine Serum, 4.5 mM glucose, 1 mM sodium syruvate, and penicillin/streptomycin, respectively. Cells were maintained in a humidified, 5% CO_2 incubator at 37^{o}C. A23187, Ionomycin free acid, actinomycin D (Calbiochem), tBHQ, and TPA (Sigma Aldrich) were dissolved in DMSO. Cyclosporin A (Calbiochem) was dissolved in EtOH.

Promoter reporter constructs and DNA transfection: pGL3-ARE mouse-ferritin H and –0.22kb mouse-ferritin H-Luc were constructed by Sma I digestion of pGL3-4.8 kb m-ferritin H [28] followed by either self ligation or insertion of double stranded ARE oligo. pBluescript SK(-)ARE-TATA human ferritin H-luciferase reporter plasmids were previously described [17]. Jurkat cells were transiently transfected via electroporation (BioRad Gene Pulser X-Cell) utilizing a manufacturer presetting for each cell type. As an internal control for transfection efficiency, 0.1 ug of pRL-EF (elongation factor promoter-renilla luciferase reporter, Promega) was simultaneously cotransfected. Following electroporation of luciferase promoter-reporter constucts into $1x10^7$ cells, cells were seeded at an initial density of 4-5 x 10^5 cells per 35 mm dish containing 2 ml culture medium. After incubation for 48 hours, and addition of 2 ml fresh RPMI media, the cells were treated with the indicated stressors for 24 hours. Preparation of cell extracts and luciferase assays were performed using Dual Luciferase Assay Reagents (Promega, Madison, WI). Firefly

luciferase expression driven by the ARE of the ferritin H gene was normalized to the Renilla luciferase activity.

Western blotting: Western blots were performed using either whole cell lysates prepared using Reporter Lysis Buffer (Promega, Madison, WI), Lysis Buffer A Solution containing 10 mM Na_2HPO_4, 150 mM NaCl, 1% TritonX100, 0.5% sodium deoxycholate, 0.1% SDS, and 0.2% Na azide, pH 7.4, or cytosolic and nuclear extracts obtained using Nuclear Extract Kit (Active Motif, Carlsbad, CA). Unless otherwise mentioned, 50 ug total protein was subjected to SDS-PAGE, and all primary antibodies were incubated overnight at 4^oC. NFAT family antibody (k-18X, SCBT) and NFATc1 specific antibody (7a6X, SCBT) were used with a working dilution of 1:500 in phosphate-buffered saline (PBS) containing 0.1% tween and 5% skim milk. Ferritin H specific antibody (H-53, SCBT) was diluted 1:1000. Secondary antibodies (anti-rabbit IgG, anti-mouse IgG (Alpha Diagnostic), and anti-Goat (Calbiochem)), were used at 1:5000 working dilutions. Signals were visualized via ECL Western Blotting Detection Reagents and ECL Advance Western Blotting Detection Reagents (Amersham-GE Healthcare).

Northern blotting: Jurkat cells were treated for 24 hours with 0, 1, 5 uM ionomycin, or an equimolar amount of DMSO vehicle. NIH3T3 cells were treated for 24 hours with 1 uM A23187 or ionomycin. In some cases, cells were pretreated with cyclosporin A. Total RNA was isolated using Tri Reagent (Invitrogen) or TriZol (Sigma). 5-15 ug total RNA was applied to a 1.1% agarose formaldehyde-containing gel. The separated RNA was blotted to a 0.45 micron nitrocellulose Protran BA85 membrane (Whatman, Schleicher & Schuell) and

ferritin H mRNA was detected using an α-^{32}P-dCTP labeled 0.9 kb fragment of ferritin H human cDNA as a probe.

Gel Retardation Assay: Nuclear and cytosolic extracts were prepared using the nuclear extraction kit from Active Motif. Binding reaction and separation of retarded bands by polyacrylamide gel electrophoresis was described previously [17]. NFAT antibody, along with wild type and mutant NFAT consensus oligonucleotides, was obtained from Santa Cruz Biotechnology.

^{35}S Translabeling /ferritin immunoprecipitation: Jurkat cells were treated with either 2.5 uM ionomycin for 0, 1, 2, 4, 8, or 24 hours, or 1, 2.5, 5 uM ionomycin, or tBHQ for 24 hours. After treatment, cells were pelleted at 1000 rpm for 5 min, and normal growth media was replaced with methione/cysteine deficient media. Simultaneously, 10 uCi/ml of ^{35}S methionine/cysteine (GE Healthcare) was added to each pellet and incubated for 1 hour. Total cell lysates were prepared with Lysis Buffer A Solution, and cleared using non-immunized rabbit serum (CAPEL) and protein A agarose (Calbiochem) overnight at 4^{o}C. ^{35}S incorporation was measured by TCA precipitation and scintillation counting. The amount of input for each immunoprecipitation was determined by adding equal radioactive counts to each immunoprecipitation reaction. 6 ul of anti-ferritin antibody (DAKO, A133) and 20 ul of protein A agarose were utilized for overnight immunoprecipitation at 4^{o}C. Finally, the resulting immunoprecipitates were subjected to SDS-PAGE as described above, and the dried gel was exposed to film at –86^{o}C for 2-5 days.

RESULTS

Calcium ionophore increases ferritin H expression in Jurkat cells. We previously demonstrated that ferritin H is induced by various oxidative stressors and during K562 erythroid differentiation by hemin [16, 17, 22, 29]. Because T-cells are exposed to high levels of ROS during activation and also may subsequently undergo apoptosis [30, 31], we investigated whether the initiating signal for T-cell activation, intracellular calcium increase, may induce ferritin H. Jurkat acute leukemic T-cells were treated with the calcium ionphore, ionomycin, 12-O-tetradecanoylphorbol-13-acetate (TPA), or TPA and ionomycin together. TPA is a tumor promoter, which also activates PKC signaling [32]. Co-treatment of TPA and ionomycin is a prototypical model for T-cell activation [33]. Treatment of Jurkat cells with 1 and 2.5 uM ionomycin resulted in a dose-dependent increase in ferritin H protein (Fig. 1a). Neither TPA nor TPA/ ionomycin induced ferritin H protein; in fact, ferritin H protein expression appears to be slightly decreased compared to control. Another phase II enzyme, glutathione s-transferase-pi (GST-pi), was unaffected by ionomycin or TPA treatment, and is shown as a loading control.

Ferritin is regulated by iron regulatory proteins (IRPs) at the translational level [27]. We previously reported that H_2O_2 transiently represses ferritin H protein translation [29]. To examine the translational regulation of ferritin by ionomycin, we performed ^{35}S methionine translabeling and ferritin immunoprecipitation. Treatment of Jurkat cells for 1, 2, 4, 8, and 24 hours resulted in enhanced translation of ferritin protein (Fig. 1b). We also observed that ferritin H protein appears to be preferentially upregulated in comparison to ferritin L. In addition, treatment with increasing concentrations up to 2.5 uM ionomycin, resulted in a

dose responsive increase in ferritin H protein synthesis. The amount of synthesized ferritin H following ionomycin treatment is similar to that of tBHQ, a prototypical phase II gene activator [34]. The highest dose of ionomycin still resulted in a modest increase in ferritin H protein synthesis in a similar manner to that observed in our initial Western blotting result. This may be due to some toxicity observed at higher concentrations of ionomycin.

In addition to ferritin translational regulation, ferritin H can also be activated at the transcriptional level [35]. To address whether or not ionomycin treatment may regulate the ferritin H gene, we exposed both Jurkat and NIH3T3 cells to either 1 or 5 uM ionomycin or vehicle control for 24 hours and assessed ferritin H mRNA by Northern blotting (Fig. 2). Ferritin H mRNA was increased by ionomycin treatment in a dose responsive manner in both Jurkat and NIH3T3 cells.

NFAT transcription factor is activated by ionomycin treatment. NFAT is one of the major transcription factors activated by increased intracellular calcium during T-cell activation. Our previous result showed that ferritin H mRNA increased; therefore, we hypothesized that the increase may be due to transcriptional activation of the ferritin H gene by NFAT. First, we confirmed that NFAT was activated by ionomycin treatment. Nuclear and cytoplasmic fractions of Jurkat cells treated with ionomycin, TPA, or TPA/ ionomycin were subjected to Western blotting to detect the translocation of NFAT to the nucleus. Both ionomycin and, to a greater extent, TPA/ionomycin treatment resulted in an increase in the amount of NFAT detected in the nuclear fraction, suggesting that NFAT is activated and available to bind to DNA. To reveal the ability of translocated NFAT to bind its target sequence the same

cellular fractions were utilized for gel retardation assay using an NFAT consensus sequence (derived from the IL-2 promoter) labeled with γ^{32}P-ATP for gel retardation assay. A shifted band was detected in the ionomycin and TPA/ ionomycin treated nuclear fractions, suggesting that the translocated NFAT is capable of binding to specific DNA target elements. A shifted band was not detected after treatment with the TPA alone, which is in agreement with our previous result that TPA does not induce ferritin H protein (Fig. 1).

Ferritin H mRNA induction following ionomycin treatment is independent of NFAT-mediated transcriptional activation. Because NFAT is activated by ionomycin and is involved in the transcription of cytokine genes during T-cell activation [1], and ferritin H mRNA was induced by ionomycin, we hypothesized that NFAT may target the ferritin H promoter for activation following ionomycin treatment. Upon examination of the mouse and human ferritin H promoter sequence, we identified two putative NFAT binding sites adjacent to the AP-1/NFE2 sites found in the antioxidant responsive element (Fig. 4a). Though the putative NFAT binding site was not an exact match, the presence of a contiguous AP-1 binding site increases the likelihood of NFAT binding because NFAT cooperatively binds and functions with AP-1 family transcription factors [35]. First, we examined whether or not protein binding to the NFAT-ARE sequence following ionomycin treatment was increased. A probe containing the putative NFAT site and AP-1/NFE2 site was used for gel retardation assay with nuclear extracts obtained from Jurkat cells treated with ionomycin. We observed no increase in the amount of protein binding after ionomycin treatment compared to the no treatment control (Fig. 4b). TPA and TPA/Ionomycin stimulation both resulted in a strong increase in the amount of protein bound to the ARE.

This is likely due to the stimulation of AP-1 transcription factor binding by TPA. This suggests that NFAT may not bind to the putative NFAT site identified in the human ferritin H ARE, since ionomycin treatment did not stimulate a protein binding increase. Next, we asked whether or not the ferritin H promoter is activated by ionomycin. We employed 5.2 kb of the upstream region of the human ferritin H promoter fused to a luciferase reporter [17] for transient transfection. Ionomycin did not activate the ferritin H promoter; it did, however, strikingly induce an NFAT-Luciferase promoter reporter construct (Fig. 4c). In addition, the antioxidant tBHQ was included as a postive control, and activated the ferritin H promoter. Taken together, these results suggest that ionomycin does not activate ferritin H transcription, and also that NFAT does not play a role in ferritin H regulation by ionomycin. It is possible that some repressor element may be present in the entire –5.2 kb region of the ferritin H gene, so we employed a wild type ARE consensus oligonucleotide inserted into a TATA-luciferase reporter for transient transfection assay. Similarly, the ARE was also not activated by ionomycin, though it was induced by tBHQ. Thus, our results suggest that ferritin H is not transcriptionally activated by ionomycin.

To confirm that NFAT is not involved in ferritin H mRNA induction, we employed cyclosporin A. Cyclosporin A is an immunosuppressant that blocks the activation of calcineurin and also prevents downstream calcium mediated signaling events. To confirm that cyclosporin A abrogates ionomycin-mediated activation of NFAT, we examined the amount of NFAT present in the nuclear fraction following treatment. NFAT was increased in the nuclear fraction after ionomycin treatment alone, but the addition of cyclosporin A completely blocked NFAT translocation (Fig. 5a). When ferritin H mRNA levels were

examined, cyclosporin A treatment actually enhanced basal expression (Fig. 5b). It did not appear to abrogate ionomycin-mediated increase in ferritin H mRNA. We also showed that cyclosporin A had no effect on ferritin L mRNA, which was decreased dose-dependently by ionomycin treatment.

Ionomycin mediated induction of ferritin H occurs via a posttranscriptional mechanism involving mRNA stabilization. Our previous results suggested that ferritin H is not transcriptionally activated by ionomycin, and indicated that ferritin H induction is independent of NFAT activation. We were interested in whether or not the increase in ferritin H mRNA following ionomycin treatment may be due to a posttranscriptional mechanism. Enhanced mRNA stability is one of the major posttranscriptional mechanisms that can increase mRNA levels. One of the main mechanisms of mRNA stabilization is the binding RNA binding proteins to target sequences in the RNA, which allows them to modulate the stability of the transcript. Upon examination of the 3'UTR of ferritin H mRNA, we identified several consecutive au-rich elements. Au-rich elements are one of the main binding sequences for proteins involved in control of mRNA stability [36]. To test whether or not ferritin H mRNA stability is enhanced by ionomycin treatment, Jurkat cells were treated with either vehicle or ionomycin, and then treated for up to 8 hours with the transcription inhibitor, actinomycin D. Ionomycin treatment prolonged ferritin H mRNA half-life and appeared to confer mRNA stabilization (Fig. 6b). Taken together, the presence of au-rich elements in the ferritin H mRNA 3'-UTR and the fact that ionomycin treatment retarded ferritin H mRNA degradation, it is likely that ionomycin posttranscriptionally regulates ferritin H, plausibly through mRNA stabilization.

DISCUSSION

T-cells are subjected to elevated intracellular calcium and increased ROS during activation [2, 30]. Our previous studies and others have demonstrated that ferritin H is an iron sequestering protein that is transcriptionally activated by a number of oxidative stress inducing compounds through an antioxidant responsive element in the far upstream region of the promoter [16, 17, 24, 29, 37]. We hypothesized that increased intracellular calcium would induce ferritin H. We observed that, indeed, ferritin H mRNA and protein are increased following treatment with calcium ionophore (Figs. 1, 2). Interestingly, the potent T-cell activating stimulus of co-treatment with TPA and ionomycin did not induce ferritin H. This may be a result of the activation of AP-1 transcription factors by TPA that are involved in the repression of the ferritin H gene, a suggestion that is supported by our finding that protein binding to the ferritin H ARE was increased by both TPA and TPA/Ionomycin treatment, but not by ionomycin alone.

NFAT is a critical transcription factor involved in the activation of cytokine genes during T-cell activation [4]. We identified a putative NFAT binding site adjacent to the AP-1/NFE2 binding site in the ferritin H ARE. Though the NFAT binding sequence homology is rather low, the presence of a contiguous AP-1 binding site enhances the potential for NFAT binding and transactivation. Fos and Jun family transcription factors enhance NFAT mediated transcriptional activation in cytokine genes [38]. Though we confirmed the translocation and transactivating potential of NFAT on a consensus binding site derived from its classical target, the IL-2 promoter, following ionomycin treatment, the 5.2kb upstream region of the ferritin H promoter was not activated in our transient transfection. In

addition, ionomycin did not activate the wild-type ARE inserted into a TATA-luciferase promoter reporter (Fig. 4c). Finally, cyclosporin A blocked NFAT activation by ionomycin, but actually enhanced basal levels of ferritin H mRNA (Fig. 5). It is possible that the inhibition of the phosphatase, calcineurin, may prevent the dephosphorylation of some transcription factors that positively regulate ferritin H transcription. Interestingly, calcineurin is inhibited by ROS [39], suggesting that inhibition of phosphatase activity is a pan-activating action that allows for the activation or maintenance of activation of many transcription factors that may be important to a stress response. In the case of NFAT, it is positively regulated by dephosphorylation by calcineurin [40]. Our results indicate that the ionomycin-mediated induction of ferritin H mRNA appears to occur through a pathway distinct from calcineurin-NFAT mediated transcriptional activation. Interestingly, ionomycin decreased ferritin L mRNA in a dose dependent manner (Fig 5b). Ferritin L also contains an ARE [41]; however, many prooxidants preferentially activate ferritin H [29]. Furthermore, ferritin H and not ferritin L confers cytoprotection against oxidative stress [42].

Since ferritin H did not appear to be transcriptionally activated by ionomycin treatment, we investigated whether or not ferritin H may be posttranscriptionally regulated. Examination of the 3'UTR of ferritin H mRNA revealed the presence of two contiguous au-rich elements, suggesting that RNA binding proteins may target ferritin H mRNA for posttranscriptional regulation. Au-rich elements are localized in the UTRs of mRNA of many short-lived transcripts, including those of immediate early genes and cytokines [36, 43]. Interestingly, IL-2 is stabilized by ionomycin treatment [44]. Recent studies have indicated that mRNA

stabilization may be one event in calcium signaling [45, 46]. When we examined the stability of ferritin H mRNA following ionomycin exposure and actinomycin D treatment, we found that the half-life of ferritin H mRNA was extended by ionomycin treatment. The overall degradation of ferritin H mRNA appeared to retarded by ionomycin, supporting our hypothesis that mRNA stabilization may be the mechanism of ferritin H induction by ionomycin. Of the RNA-binding proteins that are involved in mRNA stability and that target au-rich elements, most are involved in the destabilization of mRNA. Few, including p38, HurR, and tristetraprolin have been implicated to play a role in conferring stability to message [47-49]. The mechanism by which transcripts are stabilized is unclear. The poly-A tail does provide a degree of protection from degradation machinery and ubiquitination pathways that have been proposed to be involved in the destabilization and degradation of mRNA [50]. Future studies that elucidate the responsible RNA binding protein and mechanism of enhanced mRNA stability will provide more insight into this unique pathway of posttranscriptional regulation of the ferritin H gene in response to increased intracellular calcium.

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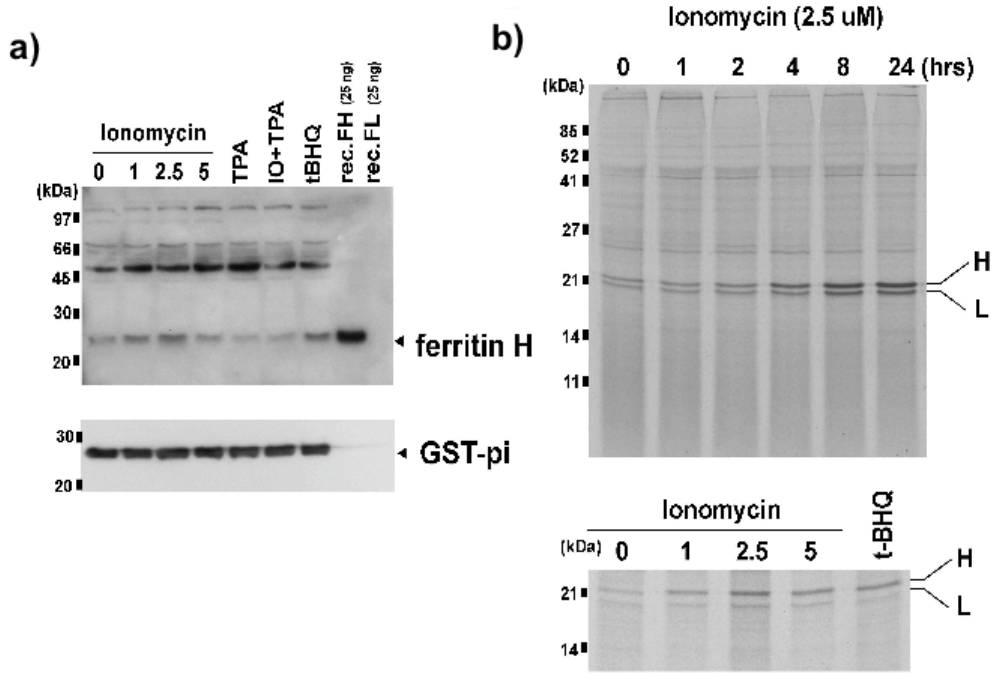

Fig. 1 Ferritin protein expression is increased by calcium ionophore treatment.
a) Jurkat cells were treated with 0, 1, 2.5 and 5 uM ionomycin (IO), TPA, TPA+IO, or tBHQ (as a positive control for ferritin H induction) for 24 hours, and whole cell extracts were subjected to Western blotting using anti-Ferritin H specific antibody. Recombinant ferritin H and L (recFH, recFL) were included to assess specificity. Ferritin H specific band is indicated with an arrow. The membranes were re-probed with anti-GST pi as a loading control. **b)** In vivo ^{35}S methionine/cysteine labeling was performed following incubation with 2.5 uM ionomycin for 0-24 hours, or 24 hours incubation with 0-5 uM ionomycin. Lysates equaling $1x10^7$ TCA insoluble counts/reaction were subjected to immunoprecipitation with anti-ferritin antibody. Ferritin H and L bands are indicated (H, L).

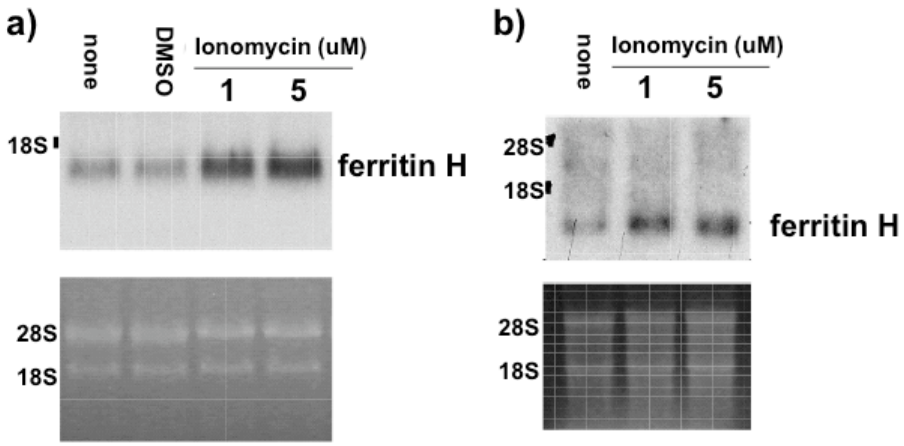

Fig. 2 Calcium ionophore treatment increases ferritin H mRNA.
Jurkat (**a**) or NIH3T3 cells (**b**) were treated for 24 hours with 0, 1, 5 uM ionomycin, or an equimolar concentration of DMSO vehicle was added. Northern blots were performed using 5-15 ug total RNA and α^{32}P-dCTP random primer labeled human ferritin H cDNA probe. The ferritin H specific band and migration of the 18 and 28s ribosomal RNA subunits are indicated. EtBr staining of RNA was performed to assess equal loading. Representative images of 3 independent experiments are shown for both (**a**) and (**b**).

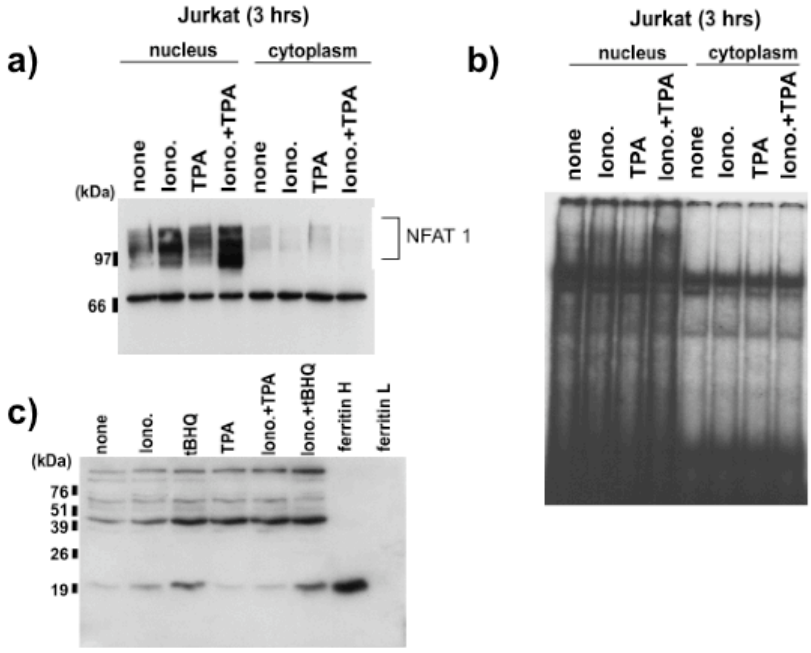

Fig. 3 NFAT is activated by calcium ionophore treatment. Nuclear and cytoplasmic fractions from Jurkat cells treated with ionomycin (IO), TPA, or ionomycin + TPA (IO+TPA) for 3 hours were prepared. **a)** Nuclear and cytoplasmic fractions were used for Western blotting with anti-NFAT antibody. **b)** Fractions were used for gel retardation assay with a γ32P-ATP labeled NFAT consensus binding sequence as a probe (see materials and methods). **c**) Jurkat cells were treated with ionomycin, TPA, tBHQ, alone or in combination as indicated for 24 hours. Whole cell extracts were prepared and ferritin protein expression was analyzed by Western blotting with anti-ferritin H antibody. Recombinant ferritin H and L are also shown.

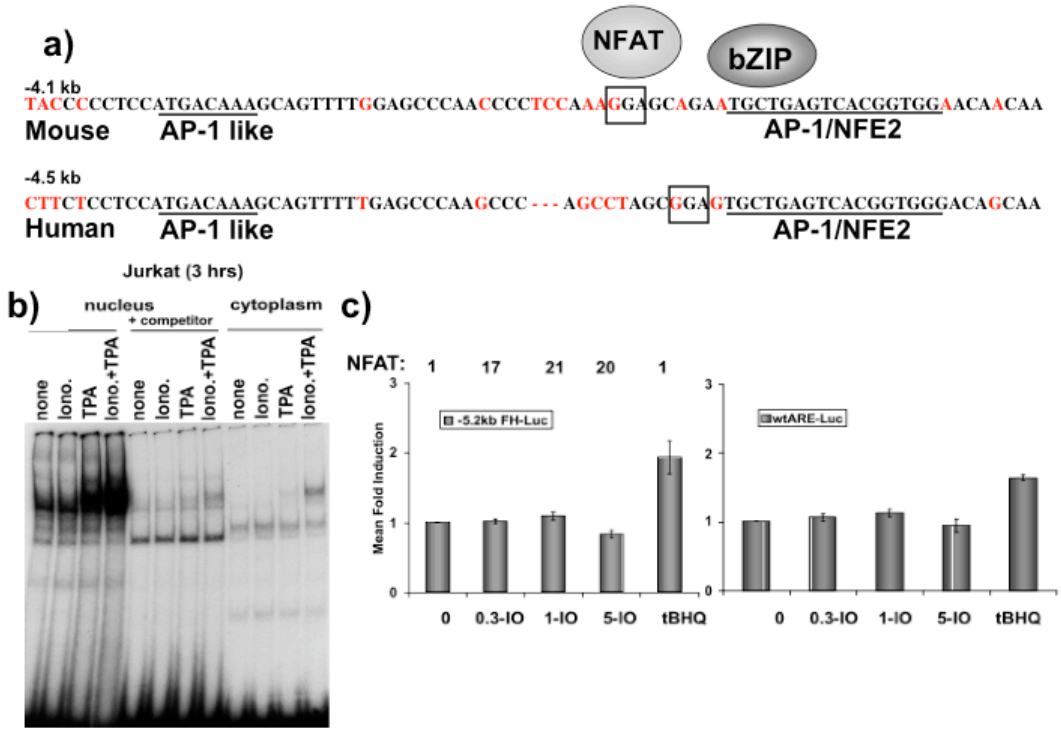

Fig. 4 NFAT is not involved in ferritin H transcriptional activation by calcium ionophore. **a**) Schematic of the human and mouse ARE sequences, including critical AP-1 like, AP-1/NFE2 sites (underlined), and putative NFAT binding sites (box). **b**) Nuclear and cytosplasmic extracts were prepared from cell treated with ionomycin (Iono), TPA, or Iono +TPA for 3 hours. 50 ug of each fraction was utilized for gel retardation assay with a γ32P-ATP labeled extended AP-1/NFE2 probe, containing the putative NFAT site. +Competitor indicates the addition of cold competitor in 50-fold excess to the binding reaction. **c**) Jurkat cells were transiently transfected with either –5.2 kb human FH-Luc, or wt ARE-Luc. Cells were allowed 24 hr recovery period and then treated with the DMSO or 0.3, 1, or 5 uM ionomycin (IO), or tBHQ (10 uM). Cell lysates were prepared and luciferase activity was assessed via luminometry with dual luciferase reagents (Promega). DMSO treated activity was set to 1. S.E.M are shown, n=6 independent experiments with duplicate samples. The relative activity of NFAT-Luc (mean fold induction) for each treatment is indicated above each respective bar at the top of the graph.

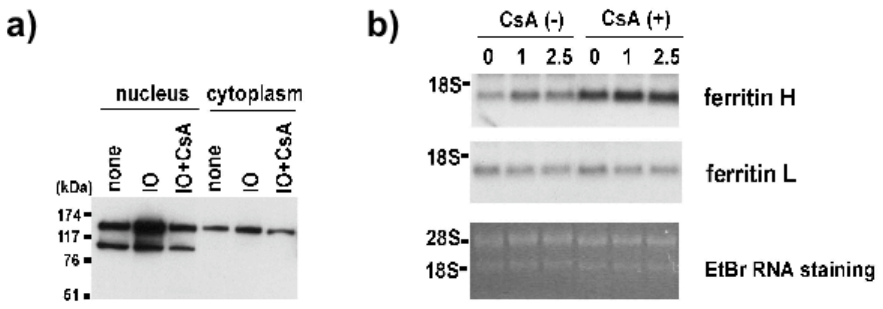

Fig. 5 Inhibition of NFAT by cyclosporin A does not block ionomycin mediated ferritin H mRNA induction. a) Nuclear and cytoplasmic fractions were prepared from Jurkat cells treated with either ionomycin or ionomycin + cyclosporin A. Western blotting for NFAT was performed to assess nuclear translocation of NFAT using an NFAT-1 specific antibody (Santa Cruz). **b**) Jurkat calls were treated with 0, 1, or 2.5 uM ionomycin with or without cyclosporin A for 24 hours. Total RNA was harvested and ferritin mRNA levels were assessed by Northern blotting, using ^{32}P-dCTP labeled human ferritin H or ferritin L cDNA probe. The specific ferritin H and L bands are indicated. EtBr staining (shown below) was performed to assess RNA loading. Representative images of 3 experiments are shown.

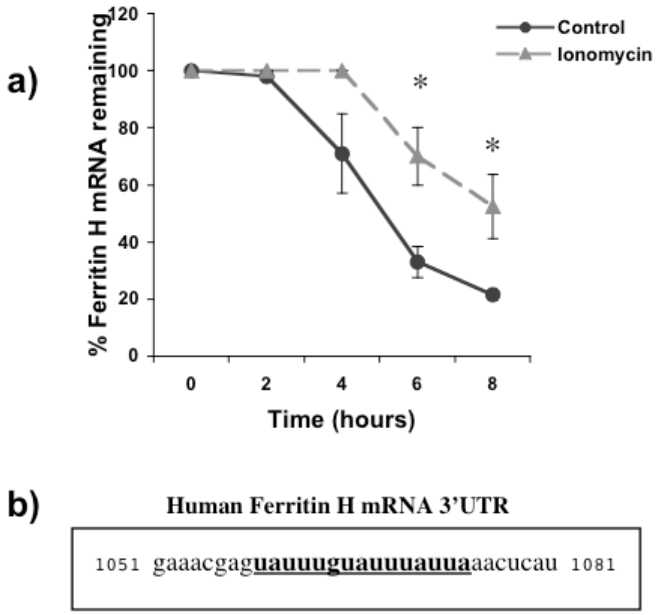

Fig. 6 Ionomycin treatment results in the stabilization of ferritin H mRNA. a) Jurkat cells were treated with 0 or 1 uM ionomycin were exposed to actinomycin D for up to 8 hours. At the indicated time points, RNA was harvested. 2-5 ug RNA was utilized for Northern blotting with a ferritin H cDNA probe. The resulting ferritin H mRNA signal was analyzed by densitometry (ImageJ) and normalized to ethidium bromide staining. S.E.Ms are shown of n=3 experiments, and * is defined as $p<0.05$, as determined by Student's t-test. **b**) A region of the human ferritin H 3'UTR is shown, putative au-rich elements are underlined.

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CONCLUSION

In the first study, I have identified a pathway of transcriptional activation of the ferritin H gene in response to the mitochondrial complex I inhibitor and neurotoxic compound, rotenone, that involves transactivation of the antioxidant responsive element (ARE) and requires reactive oxygen species (ROS). The increased ROS production and apoptosis caused by rotenone in ferritin H knockdown cells versus control cells implicate ferritin H in suppressing apoptosis purportedly by abatement of ROS production. These findings identify ferritin H transcriptional activation through the ARE as an important cytoprotective mechanism in response to mitochondrial dysfunction and ROS production by rotenone. We assessed the regulation of ferritin H by elevated intracellular calcium in the second study. Though calcium did not transcriptionally activate the ferritin H gene, it caused an elevation in ferritin H mRNA levels due to posttranscriptional mechanism, which appeared to involve mRNA stabilization.

A recent study by Pham et al. 2004 identified a role for ferritin H induction by NFkB in decreasing ROS production and blocking apoptosis in response to TNFα stimulation [1]. NFkB is an antiapoptotic protein that has been shown to suppress ROS production [2]. Calcium elevation, in addition to TNFα, activates NFkB [3]. Thus, calcium and ROS both seem to be involved in NFkB function, and thereby may indirectly modulate the expression of the ferritin H gene and other NFkB target genes. The relationship between calcium and ROS in the deleterious effects of rotenone on neuronal cells was established by recent studies [4, 5]. A role for calcium influx in rotenone-mediated apoptosis was

demonstrated by suppression of apoptosis using intracellular calcium chelators [4]. In addition, intracellular calcium concentrations in the micromolar range enhanced mitochondrial oxidative stress in the presence of rotenone [5].

Oxidative stress also may modulate the delicate calcium signaling balance. ROS can induce calcium influx from extracellular sources or from the depots in the endoplasmic reticulum [6]. H_2O_2 has been shown to cause transient calcium elevation from release of endoplasmic reticulum stores, and may be a result of oxidant-mediated induction of inositol 1,4,5-triphophate (PIP_3) [7]. Increased production of PIP_3 is a result of PI3K activation, which also leads to the subsequent activation of Nrf2. Nrf2 binding enhances transcription of ARE-containing phase II genes [8], while elevated PIP_3 levels may cause an increase in intracellular calcium [9]. Accordingly, Lee et al. reported that phosphatidylinositol 3-kinase (PI3K) activation by tBHQ is responsible for transactivation of the NADPH:quinone oxioreductase (NQO1) ARE by Nrf2 [10]. This finding is in agreement with our demonstration of enhanced Nrf2 binding to the ferritin H ARE during rotenone-mediated transcriptional activation.

Increase in cellular antioxidant potential by transcriptional activation of ARE-containing genes, like ferritin H, may represent a cytoprotective response to both the ROS and the concomitant increase in intracellular calcium. This assertion is supported by recent observations that tBHQ protects neuroblastoma cells from apoptosis resulting from oxidative stress induced by the neurotoxicant, 6-hydroxydopamine [11]. Additionally, a cytoprotective gene called DSCR1 (adapt78) has been identified [12], which is induced in

response to calcium ionophore and H_2O_2 [13]. DSCR1 is an inhibitor of calcineurin, a phosphatase that is activated by calcium and is involved in the activation of nuclear factor of activated t-cells (NFAT) and the propagation of apoptosis [14]. In a neuronal model, DSCR1 was able to protect against stress caused by calcium and H_2O_2 by inducing the antioxidant gene, superoxide dismutase (SOD1) [15]. Thus, there may be a group of adaptive genes that are capable of attenuating the ROS/calcium stress signals that lead to damage and apoptosis.

Our identification of ferritin H mRNA stabilization by calcium provides a novel mechanism of upregulation of a potential stress responsive enzyme. In addition, our findings that rotenone induces the ferritin H gene through oxidative-stress mediated transcriptional activation of the ARE and that ferritin H may abrogate ROS production and apoptosis induced by rotenone support previous evidence for the role of ARE activation in cytoprotection. The role of calcium and oxidative stress signaling in response to rotenone has clear implications in terms of understanding some of the events involved in the complex pathogenesis of Parkinson's disease; however, because of the ubiquitous nature of calcium and oxidants, the regulation of cytoprotective response genes, such as ferritin H, may be important in many other diseases involving different cell populations, including those of the immune and cardiovascular systems.

In terms of our studies on rotenone and calcium mediated regulation of ferritin H, use of intracellular calcium chelators and antioxidants to examine the causative agent in the induction of apoptosis by rotenone or calcium overload would be helpful. In addition,

further study of the upstream signaling pathways involved in the activation of Nrf2 and JunD would provide a more detailed mechanism. Furthermore, employment of antioxidants and calcium chelators to examine their effect on downstream transcription factor binding would further enhance our understanding of the critical signals involved in the stress response. Finally, mRNA protein binding studies may help elucidate the important RNA binding proteins involved in the stabilization of ferritin H mRNA in response to calcium; thus, providing more insight into this novel posttranscriptional part of the adaptive stress response. Future studies that further elucidate the detailed mechanisms of the upregulation of cytoprotective genes, such as ferritin H, will provide insight into potential sensitive events that may be involved in the development of Parkinson's disease and other diseases where oxidative stress and/or calcium may be involved. They may also provide potential therapeutic targets for modulation and chemoprevention.

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Hemin-Mediated Regulation of an Antioxidant-Responsive Element of the Human Ferritin H Gene and Role of Ref-1 during Erythroid Differentiation of K562 Cells

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Hemin-Mediated Regulation of an Antioxidant-Responsive Element of the Human Ferritin H Gene and Role of Ref-1 during Erythroid Differentiation of K562 Cells

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An effective utilization of intracellular iron is a prerequisite for erythroid differentiation and hemoglobinization. Ferritin, consisting of 24 subunits of H and L, plays a crucial role in iron homeostasis. Here, we have found that the H subunit of the ferritin gene is activated at the transcriptional level during hemin-induced differentiation of K562 human erythroleukemic cells. Transfection of various 5′ regions of the human ferritin H gene fused to a luciferase reporter into K562 cells demonstrated that hemin activates ferritin H transcription through an antioxidant-responsive element (ARE) that is responsible for induction of a battery of phase II detoxification genes by oxidative stress. Gel retardation and chromatin immunoprecipitation assays demonstrated that hemin induced binding of cJun, JunD, FosB, and Nrf2 b-zip transcription factors to AP1 motifs of the ferritin H ARE, despite no significant change in expression levels or nuclear localization of these transcription factors. A Gal4-luciferase reporter assay did not show activation of these b-zip transcription factors after hemin treatment; however, redox factor 1 (Ref-1), which increases DNA binding of Jun/Fos family members via reduction of a conserved cysteine in their DNA binding domains, showed induced nuclear translocation after hemin treatment in K562 cells. Consistently, Ref-1 enhanced Nrf2 binding to the ARE and ferritin H transcription. Hemin also activated ARE sequences of other phase II genes, such as GSTpi and NQO1. Collectively, these results suggest that hemin activates the transcription of the ferritin H gene during K562 erythroid differentiation by Ref-1-mediated activation of these b-zip transcription factors to the ARE.

Iron is an indispensable element for a variety of cellular functions in metabolism, growth, and differentiation (36). Iron serves as a constituent of vital proteins, including ribonucleotide reductase and many other heme proteins, such as mitochondrial cytochromes, cytochrome P450 enzymes, and hemoglobin. In particular, during the process of maturation of erythroid cells, a sufficient amount of iron should be supplied for hemoglobinization under a coordinated partitioning of intracellular iron levels. However, an excess amount of intracellular free iron is harmful to the cells because iron can catalyze formation of reactive oxygen species through the Fenton reaction (44, 51). Therefore, intracellular iron levels should be tightly regulated by storing excess iron in a nontoxic but bioavailable form for supply upon metabolic requirement for hemoglobinization.

Ferritin is the major cellular iron storage protein that plays a role in the storage and partitioning of iron for intracellular use (5, 64). Ferritin is composed of 24 subunits of heavy chains (H) and light chains (L), with varied H-to-L ratios depending on types of tissues and their physiological conditions (26). Ferritin synthesis is regulated at both transcriptional and translational levels (67). Iron induces ferritin synthesis at a translational level through regulation of iron regulatory proteins and iron responsive element interaction in the 5′ untranslated region of ferritin mRNAs (28, 58, 65).

In contrast to the well-characterized translational mechanism of ferritin synthesis by iron, molecular mechanisms of transcriptional regulation of ferritin genes remain to be fully elucidated. Recently, our and other studies revealed that transcriptional regulation of the human ferritin H gene is regulated through at least two independent enhancer elements. One is a proximal *cis*-acting element containing a CCAAT motif approximately 60 bp and 5′ from the transcription initiation site of the human ferritin H gene (9, 41). This CCAAT element, serving as a basal enhancer of ferritin H transcription that contributes to tissue-specific expression (10) as well as upregulation of ferritin H during differentiation (41), is regulated by NF-Y transcription factors and transcriptional coactivators p300/CBP and PCAF (10, 25). Another regulatory element for ferritin H transcription is a far-upstream enhancer we have recently identified which serves as an antioxidant-responsive element (ARE) responsible for oxidative stress-mediated activation of the ferritin H gene (68). An ARE, also identified in the 5′ flanking region of various phase II detoxification genes such as glutathione *S*-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1), regulates transcriptional activation of these phase II genes in cells exposed to a wide variety of xenobiotics and reactive metabolites (31, 49). We have recently found that the human ferritin H ARE is composed of two copies of bidirectional AP1 motifs to which basic-leucine zipper (b-zip) transcription factors, such as JunD and

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NFE2-related factor 2 (Nrf2), can bind and which activate ferritin H transcription under oxidative conditions (68).

K562 human erythroleukemia cells have offered a great experimental system to study molecular mechanisms of erythroid differentiation (8). A number of reagents, including chemotherapeutic drugs, have been shown to induce K562 erythroid differentiation (59). K562 differentiation turns on hemoglobin synthesis and diminishes growth potential. It was demonstrated that treatment of K562 cells with hemin (ferriprotoporphyrin IX) induced synthesis of ferritin proteins at both transcriptional (61, 73) and translational levels (42). The hemin-mediated translational activation of ferritin synthesis was blocked by iron chelator, deferoxamine, probably due to chelating iron liberated from hemin in the cells (55). However, the hemin-mediated increase in ferritin mRNA was not blocked by deferoxamine treatment (42), suggesting that mechanisms of hemin-induced ferritin mRNA in K562 cells are independent of the amount of intracellular chelatable iron.

Given several lines of evidence showing that oxidative stress is involved in chemically induced differentiation of K562 cells (18, 47), we have tested our hypothesis that ferritin mRNA induction during hemin-mediated K562 differentiation is transcriptionally regulated through the far-upstream ARE enhancer element. In this study, we have found that (i) a hemin-responsive element of the ferritin H gene is identical to ARE, (ii) hemin treatment induced binding of cJun, JunD, FosB, and Nrf2 transcription factors to the ferritin H ARE, and (iii) redox factor 1 (Ref-1) is involved in the activation of ferritin H transcription through the ARE.

MATERIALS AND METHODS

Cell culture. K562 human erythroleukemia cells were purchased from the American Type Culture Collection. They were cultured in RPMI 1640 medium supplemented with 0.3 g/liter glutamine, 25 mM HEPES, and 10% fetal calf serum (Mediatech) at 37°C in a humidified 5% CO_2 atmosphere. Hemin was purchased from Fluka BioChemika and dissolved in 0.1 M NaOH. HMBA (*N,N'*-hexamethylenebisacetamide), TPA (12-*O*-tetradecanoylphorbol-13-acetate), and t-BHQ (*tert*-butylhydroquinone) were from Sigma.

Plasmids and DNA transfection. pBluescript SK(−)−5.2kb h-ferritin H-luciferase (where −5.2kb human ferritin H-luciferase means that this construct contains a 5.2-kb upstream region [5.2-kb DNA fragment] from the transcription start site of the ferritin H gene) as well as most deletion and mutation reporter plasmids have been described elsewhere (68). pBluescript SK(−)−4.5kb h-ferritin H ARE mutant plasmids were constructed by PCR-mediated mutagenesis using the mutated ARE primers shown in Fig. 3. Human NQO1 ARE-luciferase and rat GST-pi–luciferase plasmids were constructed by ligation of double-strand oligonucleotides of 5'-AAATCGCAGTCACAGTGACTCAGCAGAATC-3' and 5'-CAAAAGTAGTCAGTCACTATGATTCAGCAACAAA-3' (31), respectively, into the minimum promoter of −0.03kb ferritin H-TATA–luciferase. pRc/CMVJunD was constructed as described previously (68). pCMVNrf2 was constructed by digestion of pOBT7Nrf2 (clone ID 4548874; Invitrogen) with BamHI and XhoI, and the resultant Nrf2 cDNA was cloned into the pCMV vector. Transient DNA transfection into K562 cells was carried out by electroporation (Xcell; Bio-Rad) with an optimized preset condition by Bio-Rad for K562 cells (exponential decay, 1,000 μF; 155 V; 100-μl cell suspension in a cuvette with a 0.2-cm gap). After electroporation of luciferase reporters into 5×10^6 to 10×10^6 K562 cells, they were plated at a density of 4×10^6 to 5×10^5 cells per 60-mm plate containing 4 ml of the culture medium. As a transfection internal control, 0.1 μg of pRL-CMV (Promega) or pRL-EF (elongation factor promoter) was simultaneously cotransfected. After incubation for 20 to 24 h, the cells were treated with various concentrations of hemin for 24 h. In some experiments, t-BHQ treatment was also included as a positive control of ferritin H ARE activation. Ref-1 cDNA was kindly provided by T. Curran (St. Jude Children's Research Hospital, Memphis, TN). Preparation of cell extracts and luciferase assays were performed using dual-luciferase assay reagents (Promega). Firefly luciferase expression driven by the ferritin H gene was normalized by *Renilla* luciferase activity.

Gal4 reporter system and plasmids. Four micrograms of pFA2-cJun, -JunD, or -FosB, a *trans*-activator protein fused to the Gal4-DNA binding domain (Gal4DBD), was electroporated into 1×10^7 K562 cells together with 1 μg of pFL-Luc reporter containing five direct repeats of the yeast Gal4 binding site that controls expression of the luciferase gene from *Photinus pyralis* (Stratagene). Transfected cells were divided into six plates of 35-mm dishes and incubated for 24 h. Cells were then treated with 25 μM or 50 μM of hemin for 24 h and subjected to luciferase reporter assays (Promega).

Gel retardation assay. Preparation of nuclear extracts, binding reactions, and separation of retarded bands by polyacrylamide gel electrophoresis have been described previously (71). All antibodies used in gel supershift assays were purchased from Santa Cruz Biotechnology, Inc.

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were carried out according to Upstate Biology's protocol for the ChIP assay kit with some minor modifications. Briefly, a total of 1×10^6 to 4×10^6 K562 cells/100-mm plate were treated with 50 μM hemin for 4 h, 1 day, and 3 days, followed by chromatin cross-linking and preparation of cell lysates using the ChIP assay kit (Upstate Biology). DNA in the lysate (200 μl) was sheared by sonication (Sonic Dismembrator model 100, power setting 2; Fisher Scientific), with 12 cycles of 10-second pulses and 20-second intervals. Approximately 1/10 aliquots of cell lysate were immunoprecipitated with 1 μg each of antibodies (anti-ATF1, sc-243X; anti-CREB, sc-240X; anti-Nrf2, sc-722X; anti-JunB, sc-46X; anti-c-Jun, sc-1694X; anti-JunD, sc-074X; anti-FosB, sc-48X; anti-Jun family, sc-044X; and anti-Fos family, sc-253X) (Santa Cruz Biotechnology). Quantitative PCR with a pair of primers giving rise to a 0.15-kb DNA fragment and gel electrophoresis were performed as described previously (68).

Western blotting. Total cell lysates or nuclear and cytoplasmic fractions were loaded on 10% or 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer of separated proteins to an Immobilon-P (Millipore) polyvinylidene difluoride membrane and incubation at 4°C overnight with one of the following antibodies: anti-ferritin H, anti-c-Jun, anti-JunD, anti-FosB, anti-Nrf2, anti-hemoglobin gamma, anti-Ref-1 (all from Santa Cruz Biotechnology), or anti-fetal hemoglobin (Calbiochem). Cell fractionation was carried out using a nuclear extract isolation kit (Active Motif), and purity of nuclear and cytoplasmic fractions was verified by Western blotting with anti-histone H1 (Santa Cruz Biotechnology) or anti-lactate dehydrogenase (LDH; Chemicon). Recombinant human ferritin H and L were purchased from Calbiochem.

RESULTS

Transcriptional activation of ferritin H during hemin-mediated erythroid differentiation of K562 cells. To investigate hemin-mediated induction of the ferritin H gene during erythroid differentiation, we first measured expression of ferritin H mRNA and protein during a 5-day incubation period in K562 cells. Northern blotting experiments showed that hemin treatment increased ferritin H mRNA in a dose-dependent manner, with peaking between 1 and 3 days after the treatment (Fig. 1a). The inducing effect on ferritin H mRNA appeared to be specific to hemin because HMBA, a differentiation inducer of mouse erythroleukemia (MEL) cells (43), or TPA, an inducer of megakaryotic differentiation of K562 cells (56), failed to induce ferritin H mRNA (Fig. 1a). Induction of ferritin H protein synthesis was also detected by Western blotting, reaching a maximum 1 day after hemin treatment and maintaining the high expression level until day 5 (Fig. 1b). As an erythroid differentiation marker, we monitored fetal hemoglobin expression in K562 cells (8) using two different antibodies in which hemin treatment induced synthesis of hemoglobin on day 1 and sustained the high expression level for 5 days (Fig. 1b).

We next asked whether a hemin-mediated increase in ferritin H mRNA is regulated at a transcriptional level. To address this question, we transiently transfected a 5.2kb 5' human ferritin H-luciferase reporter plasmid into K562 cells, followed by hemin treatment for 1 to 3 days, and measured luciferase

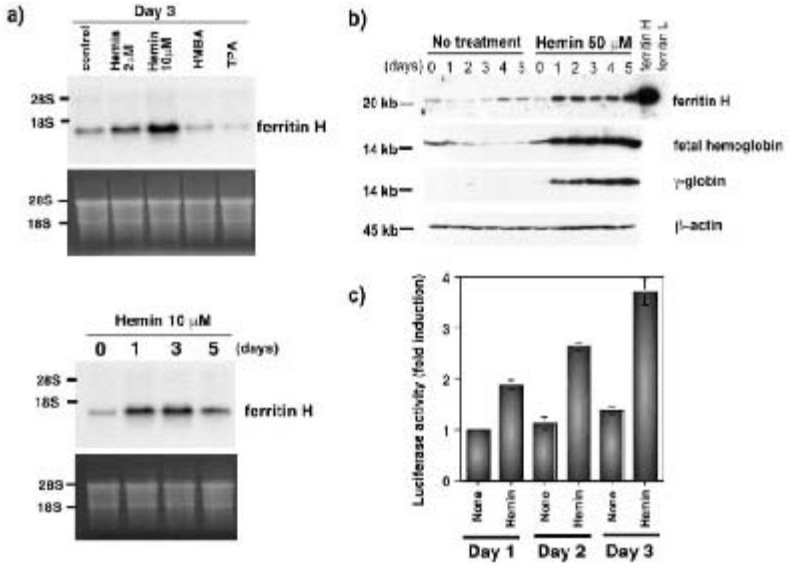

FIG. 1. Ferritin H is induced during hemin-mediated erythroid differentiation of K562 cells. (a) Total RNA was isolated from K562 cells treated with 2 μM hemin, 10 μM hemin, 5 mM HMBA, or 50 ng/ml TPA for 3 days (top) or 10 μM hemin for 1, 3, and 5 days (bottom). Ten micrograms of RNA was subjected to Northern blot analysis hybridized with human ferritin H cDNA. An equal amount of RNA loading was confirmed by staining RNA with ethidium bromide. Positions of 28S and 18S rRNA are indicated. (b) K562 cells were incubated for 1 to 5 days with or without 50 μM hemin, and 30 μg of protein from each cell lysate was separated on 10% polyacrylamide/sodium dodecyl sulfate gel for detection of ferritin H, fetal hemoglobins, γ-globin, and β-actin. Recombinant ferritin H and L proteins (50 ng) were loaded to confirm the specificity of the anti-ferritin H antibody. (c) K562 cells (5×10^6) were electroporated with 10 μg of −5.2kb h-ferritin H–luciferase plus 1 μg of pRL-CMV as an internal control of transfection and treated with 50 μM hemin for 1 to 3 days. Expression of firefly luciferase was normalized by that of *Renilla* luciferase, and the value for day 1 without treatment was set at 1.0. The results from three independent experiments are shown as means ± standard errors.

expression levels. As shown in Fig. 1c, hemin treatment activated expression of luciferase driven by the 5.2kb human ferritin H 5′ regulatory region, indicating that hemin transcriptionally activates the human ferritin H gene through a hemin-responsive enhancer element in the 5.2kb region.

Role of the ferritin H ARE in hemin-mediated transcriptional activation. In order to identify the hemin-responsive element in the human ferritin H gene, we further performed luciferase reporter assays in K562 cells using several deletion constructs of the human ferritin H gene. In contrast to hemin-mediated induction of luciferase driven by the −5.2kb ferritin H gene, luciferase reporters containing −4.0kb, −0.15kb, and −0.03kb TATA all failed to be induced by hemin treatment (Fig. 2a). The −0.15kb luciferase, which contains the CCAAT element (9, 41), showed higher basal expression than the TATA-only reporter but did not respond to hemin treatment. t-BHQ, an activator of ARE in the ferritin H gene (68, 70) and other phase II genes (38), also activated expression of only −5.2kb ferritin-luciferase (Fig. 2a) These results indicate that the hemin-responsive element is located in the region between −5.2kb and −4.0kb of the human ferritin H gene.

Recently, we identified an ARE, located 4.3kb 5′ from the transcription start site, that was responsible for oxidative stress-mediated transcriptional activation of the human ferritin H gene (68). The ferritin H ARE comprises two copies of bidirectional AP1 motifs (68). Since (i) oxidative stress was shown to be involved in erythroid differentiation of K562 cells (18, 47), (ii) hemin induces oxidative stress (54), and (iii) the human ferritin H ARE (at the −4.5kb region) is within the −5.2kb to −4.0kb region in which we identified a hemin-responsive element (Fig. 2a), we hypothesized that the hemin-responsive element is identical to the ARE. To test this possibility, we transiently transfected the ARE(+)- or ARE(−)-luciferase reporter plasmid into K562 cells and measured luciferase expression after treatment with hemin or t-BHQ. The −4.5kb ferritin H-luciferase, which contains the intact ferritin H ARE, was activated by hemin or t-BHQ treatment; however, the −4.4kb ferritin H-luciferase lacking the ARE sequence showed lower basal expression, with no response to hemin treatment (Fig. 2b). These results suggest that the ARE is involved in hemin-mediated transcriptional activation of the ferritin H gene.

To confirm this observation, we then inserted the ARE sequence into the minimum ferritin H-TATA promoter reporter plasmid and tested whether ARE alone is sufficient for hemin-mediated transcriptional activation in K562 cells. As shown in Fig. 3a, insertion of ARE made the minimum TATA reporter responsive to hemin as well as t-BHQ to induce luciferase expression. The human ferritin H ARE contains two AP1

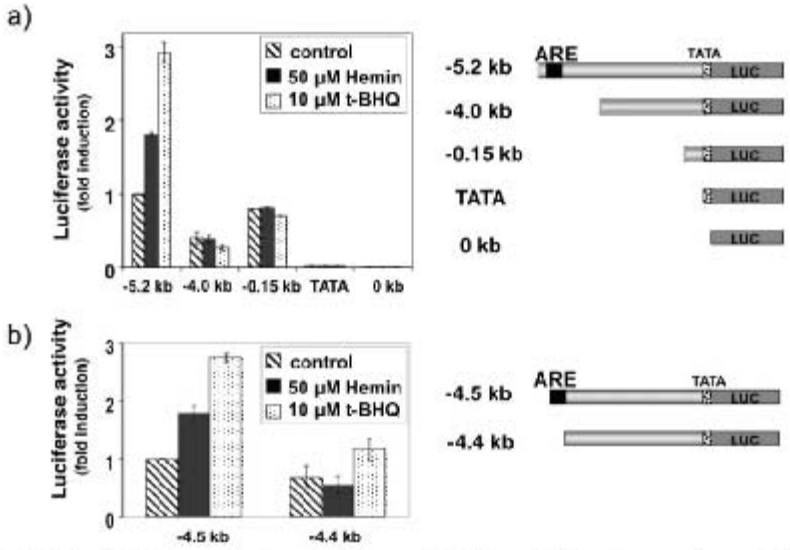

FIG. 2. Characterization of a hemin-responsive element in the human ferritin H gene. (a) Ten micrograms of human ferritin H-luciferase reporter plasmids (−5.2kb, −4.0kb, −0.15kb, TATA, or 0kb) together with 20 ng of pRL-EF was transfected into K562 cells (5×10^6 cells) by electroporation. Hemin (50 μM) or t-BHQ (10 μM) was added 1 day after electroporation and incubated for 24 h. The transfected cells were harvested to measure firefly and *Renilla* luciferases, and expression of firefly luciferase was normalized by *Renilla* luciferase expression. The luciferase activity in cells transfected with −5.2kb ferritin H reporter plasmid with no treatment (control) was defined as 1.0, and the results from four independent experiments are shown as means ± standard errors. (b) K562 cells were transfected with 10 μg of the −4.5kb luciferase reporter (+ARE) or 10 μg of the −4.4kb luciferase reporter (−ARE) by electroporation. Twenty-four hours after transfection, cells were treated with 50 μM hemin or 10 μM tBHQ or left untreated for 24 h, and cell lysates were collected to perform the luciferase assay. To obtain induction in luciferase activity, luciferase expression in extracts obtained from cells transfected with the −4.5kb luciferase reporter with no treatment (control) was defined as 1.0. The results from five independent experiments are shown as means ± standard errors.

motifs (Fig. 3), both of which are essential for the function of ARE to respond to oxidative stressors, such as H_2O_2 and t-BHQ (68). To further assess the role of the ARE in hemin-mediated ferritin H transcriptional activation, we first introduced mutations in two AP1 motifs in the ferritin H ARE, cloned one copy of each AP1 mutant into a minimum TATA-luciferase reporter and tested each motif's response to hemin treatment. Mutations in either the AP1-like motif or AP1/NFE2 motif or mutations in both sites in the ferritin H ARE almost completely abolished hemin-mediated activation (Fig. 3b). Similarly, we introduced the same mutations in the AP1 motifs with the entire 4.5kb region of the 5′ ferritin H gene and tested their response to hemin treatment. In this context, a mutation in either one of the two AP1 motifs partially impaired the hemin response, but double mutations completely abolished the hemin response in addition to significantly decreasing the basal expression levels (Fig. 3c). Collectively, these results confirmed that the hemin-responsive element is identical to the ARE of the human ferritin H gene.

Alterations in binding of b-zip family transcription factors to the ferritin H ARE by hemin treatment. To elucidate the molecular mechanism by which hemin activates transcription of the human ferritin H gene through the ARE, we performed gel mobility shift assays using nuclear extracts isolated from hemin-treated K562 cells. As shown in Fig. 4a, total protein binding to the AP-1/NFE2 site of the ferritin H ARE was increased following treatment with hemin or t-BHQ. The addition of unlabeled competitor oligonucleotide inhibited the bands that were increased by hemin treatment, indicating that the increase in protein binding was specific to the AP-1/NFE2 sequence. Next, we examined Jun, Fos, and Nrf2 family members to determine which b-zip family members comprised the ARE binding complex following hemin treatment. In gel supershift assays, both Jun and Fos family antibodies induced a supershift (Fig. 4b), indicating that transcription factors from these families bind to the ARE in vitro; however, we were unable to detect clear Nrf2 binding to the ARE using this method before or after hemin treatment (Fig. 4b).

We then carried out ChIP assays to investigate the binding of specific transcription factors to the ARE in vivo. As shown in Fig. 4c, hemin treatment for 4 h induced the binding of cJun, JunD, FosB, and Nrf2 to the ferritin H ARE, whereas JunB binding was diminished. The antibodies we used against ATF1, CREB, cFos, Fra1, and Fra2 to the ARE did not show evidence of binding to the ferritin H ARE before or after hemin treatment. Increased Nrf2 and JunD binding to the ARE was maintained until later time points of day 1 and day 3 (Fig. 4d). Cotransfection of Nrf2 and JunD together with the −4.5kb

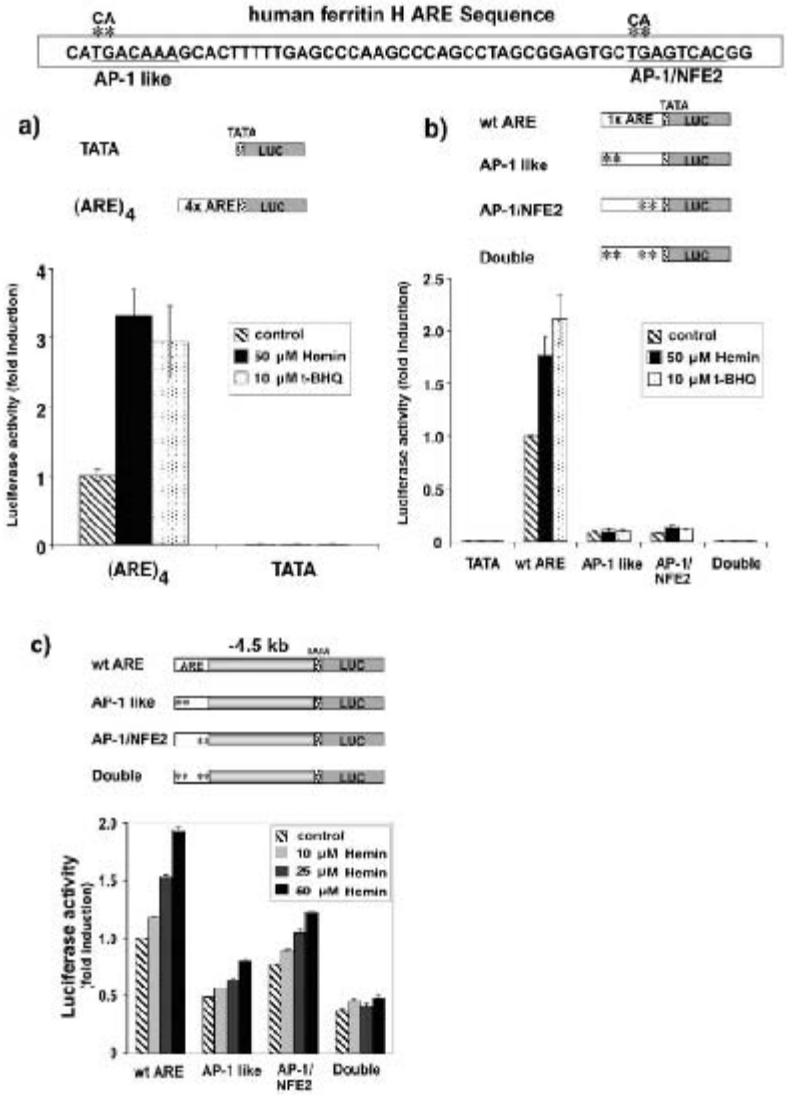

FIG. 3. Hemin activates transcription of the ferritin H gene through the ARE. K562 cells were cotransfected via electroporation with (a) 10 μg of −0.03kb ferritin H-TATA–luciferase (TATA) or insertion of four copies of the ARE in −0.03kb ferritin H-TATA–luciferase (4× ARE), (b) one copy of wild-type ARE (wt), AP-1-like mutant, AP-1/NFE2 mutant, or double mutant (see schematic), or (c) wild-type or each mutant ARE in the −4.5kb h-ferritin H-luciferase reporter, along with 20 ng of pRL-EF as an internal control. Cells were treated with 50 μM hemin or 10 μM t-BHQ (a and b) or 10, 25, and 50 μM hemin (c) for 24 h, and the resulting luciferase activity was assessed via luminometry. Induction was determined by setting 4× ARE/control (a), single-copy wild-type ARE/control (b), and −4.5kb wild-type ARE/control (c) at 1.0. The means ± standard errors from three independent experiments are shown.

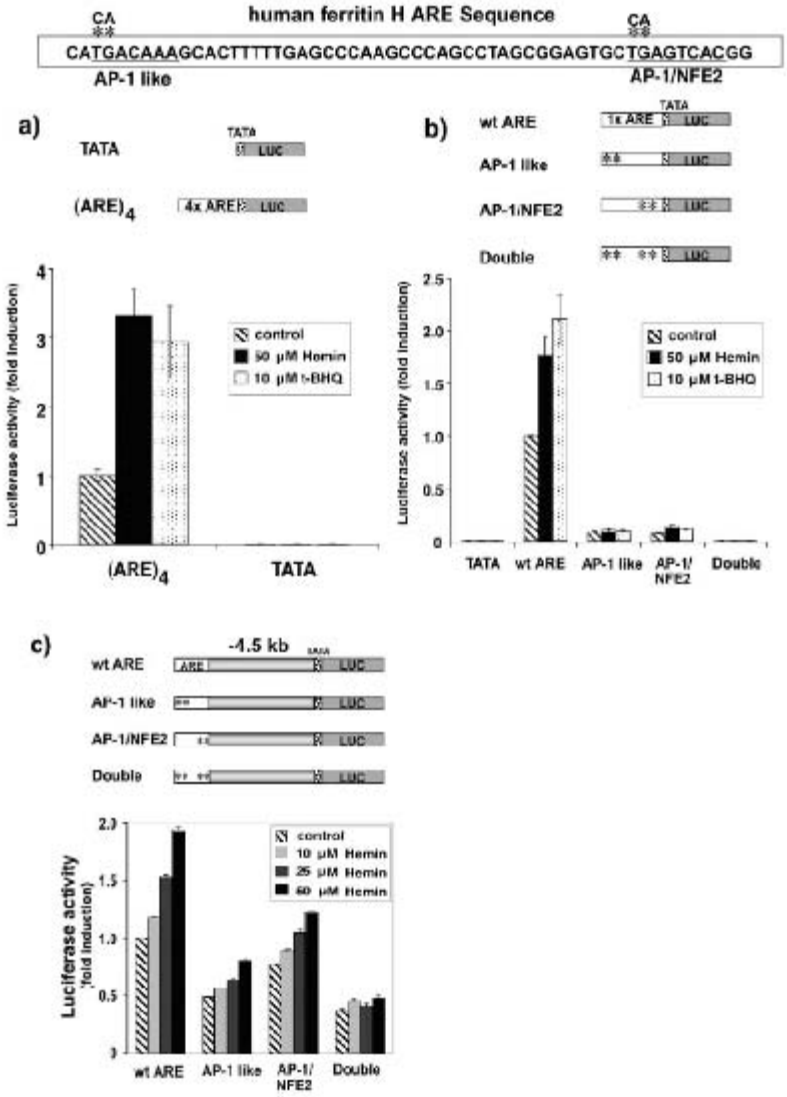

FIG. 3. Hemin activates transcription of the ferritin H gene through the ARE. K562 cells were cotransfected via electroporation with (a) 10 μg of −0.03kb ferritin H-TATA–luciferase (TATA) or insertion of four copies of the ARE in −0.03kb ferritin H-TATA–luciferase (4× ARE), (b) one copy of wild-type ARE (wt), AP-1-like mutant, AP-1/NFE2 mutant, or double mutant (see schematic), or (c) wild-type or each mutant ARE in the −4.5kb h-ferritin H-luciferase reporter, along with 20 ng of pRL-EF as an internal control. Cells were treated with 50 μM hemin or 10 μM t-BHQ (a and b) or 10, 25, and 50 μM hemin (c) for 24 h, and the resulting luciferase activity was assessed via luminometry. Induction was determined by setting 4× ARE/control (a), single-copy wild-type ARE/control (b), and −4.5kb wild-type ARE/control (c) at 1.0. The means ± standard errors from three independent experiments are shown.

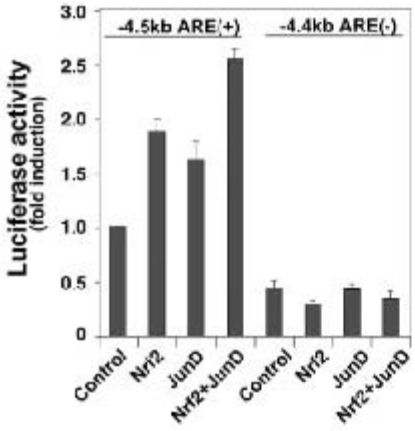

FIG. 5. Nrf2 and JunD activate the human ferritin H ARE. K562 cells were transfected with 1 μg of −4.5kb ferritin H-luciferase reporter (+ARE) or −4.4kb ferritin H-luciferase reporter (−ARE) together with either 0.5 μg of pCMVNrf2, 1 μg of pRc/CMVJunD, or 0.5 μg of pCMVNrf2 plus 1 μg of pRc/CMVJunD by electroporation. pRL-null plasmid (0.2 μg) was simultaneously transfected as an internal transfection control. The total amount of plasmid DNA in each transfection was equalized to 2.7 μg by adding pCMV or pRc/CMV empty vector. Forty-eight hours after transfection, cells were harvested for dual-luciferase assays. Expression of firefly luciferase was normalized by *Renilla* luciferase expression, and the value of the −4.5kb ARE(+) luciferase reporter with empty vector (control) was defined as 1.0. The results from five independent experiments are shown as means ± standard errors.

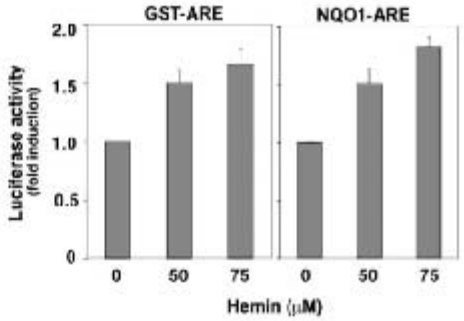

FIG. 6. Hemin activates NQO1 and GST AREs. One microgram of GSTpi-ARE–luciferase or NQO1-ARE–luciferase was cotransfected into K562 cells along with 20 ng of pRL-EF as an internal control. Cells were treated with 50 or 75 μM hemin for 24 h, and the resulting luciferase activity was measured. Induction was determined by setting the control at 1.0, and the means ± standard errors from six independent experiments are shown.

and probably other ARE-regulated phase II antioxidant genes.

Role of Ref-1 in hemin-mediated activation of ferritin H transcription through the ARE. To elucidate the activation mechanism of these b-zip transcription factors, we first employed a Gal4 reporter assay to assess posttranslational activation by hemin treatment. A fusion protein of Gal4DBD-JunD, -cJun or -FosB was expressed in K562 cells transiently cotransfected with a Gal4DBD-luciferase reporter and then treated with hemin. These Gal4DBD fusion transcription factors significantly activated the expression of luciferase; however, 25 μM or 50 μM hemin failed to further increase luciferase expression (Fig. 7a), suggesting that posttranslational activation of these transcription factors by way of, for instance, phosphorylation or sumoylation may not be the principal mechanism of the hemin-mediated activation. Since the increase in DNA binding of these transcription factors was observed in our gel shift and ChIP assays (Fig. 4), we then asked whether accumulation of these transcription factors in the nucleus is induced after hemin treatment. K562 cells treated with hemin for 4 h were subjected to the preparation of cytoplasmic and nuclear fractions, and Western blotting was performed to detect endogenous JunD, cJun, FosB, and Nrf2 expression levels. As shown in Fig. 7b and c, none of these transcription factors showed increased expression in nuclear fractions. Since Nrf2 has been shown to be the major transcription factor in regulating ARE (32, 35, 49), we also transiently transfected Nrf2 into K562 cells and carried out similar Western blotting, in which endogenous Nrf2 as well as transfected Nrf2 did not show clear nuclear accumulation after hemin treatment (Fig. 7c). Taken together, we concluded that increased binding of these transcription factors to the ferritin H ARE after hemin treatment is not due to a dynamic alteration of transcription factors per se but may require a regulatory molecule for their enhanced DNA binding capacities.

Growing evidence has demonstrated that redox regulation of protein-protein and protein-nucleic acid interactions is an important cellular defense mechanism under oxidative stress conditions (48). Stress-mediated activation of AP1 transcription factors is subject to redox regulation by Ref-1 that enhances DNA binding of AP1 via reduction of a conserved cysteine residue in the DNA binding domain (75). We therefore tested whether Ref-1 is involved in hemin-mediated ferritin H ARE activation. Ref-1 was transiently cotransfected into K562 cells together with the wild-type ARE- or mutant ARE-luciferase reporter plasmid (ARE insertion either in the minimum TATA reporter [Fig. 8a, left panel] or in the entire 4.5kb 5′ ferritin H reporter [Fig. 8a, right panel]), followed by treatment with hemin for 24 h. Ref-1 alone increased luciferase expression driven by wild-type ARE but not by mutant ARE (Fig. 8a). In the same experiment, nuclear and cytoplasmic fractions were prepared from K562 cells treated with 50 μM hemin for 4 h, and Western blotting was carried out to detect Ref-1 expression in each fraction. As shown in Fig. 8b, endogenous Ref-1 in the cytoplasm of control vector-transfected K562 cells was decreased after hemin treatment, resulting in a concomitant increase of Ref-1 in the nuclear fraction. In Ref-1-transfected K562 cells, overexpressed Ref-1 in the cytoplasm was slightly decreased in response to hemin treatment, not as significant as the endogenous Ref-1 translocation, and slightly increased Ref-1 was observed in the nucleus (Fig. 8b). t-BHQ treatment also induced nuclear translocation of Ref-1 in K562 cells (Fig. 8b). We then asked whether Ref-1

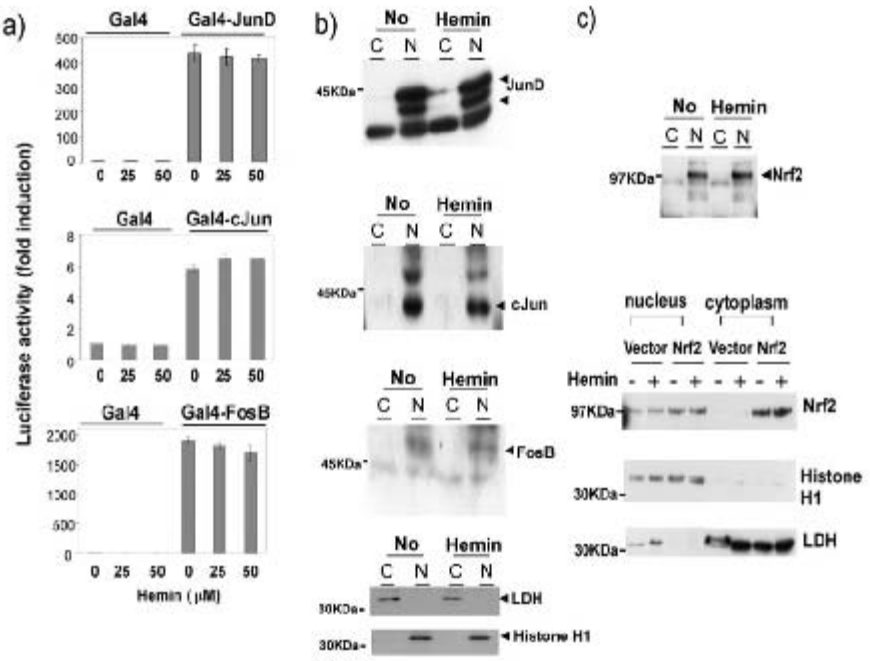

FIG. 7. Hemin and posttranslational regulation of JunD, c-Jun, FosB, or Nrf2 transcription factors. (a) A Gal4 reporter system was utilized to estimate c-Jun, JunD, or FosB activation in hemin-treated K562 cells. Four micrograms of pFA2-cJun, -JunD, or -FosB fused to Gal4DBD was electroporated into K562 cells together with 1 μg of pFL-Luc reporter containing five direct repeats of the yeast Gal4 binding element and 20 ng of pRL-EF as an internal control. Twenty-four hours after transfection, cells were treated with 25 or 50 μM of hemin for 24 h and subjected to luciferase reporter assays. The induction in Gal4DBD transfectants without treatment was defined as 1.0. The means ± standard errors from three independent experiments are shown. (b) K562 cells (4×10^6) were treated with 50 μM hemin for 4 h, and cytoplasmic (C) and nuclear (N) fractions were prepared. Fifty micrograms of each fraction was subjected to Western blotting using anti-JunD, anti-cJun, or anti-FosB. Arrowheads in the panel indicate respective transcription factor bands. (c) (Top panel) K562 cells (4×10^6) were treated with 50 μM hemin for 4 h, and cytoplasmic (C) and nuclear (N) fractions were prepared. Fifty micrograms of each fraction was subjected to Western blotting using anti-Nrf2 antibody. (Bottom panel) K562 cells (4×10^6) transfected with pCMV vector or pCMVNrf2 were similarly subjected to Western blotting for Nrf2, followed by histone H1 Western blotting (using the same membrane) and LDH Western blotting (using the same samples but a different blot) for the assessment of purity of cytoplasmic and nuclear fractions.

induces binding of Nrf2 to the ferritin H ARE. To address this question, we transiently transfected Ref-1 into K562 cells and performed a ChIP assay to assess the role of Ref-1 in endogenous Nrf2 binding to the ferritin H ARE. Our results show that Ref-1 enhances Nrf2 binding to the ferritin H ARE in vivo (Fig. 8c). These results suggest that Ref-1 is, at least in part, involved in basal expression of the ferritin H gene and hemin-mediated activation of ferritin H transcription through increased DNA binding of these b-zip transcription factors to the ARE through redox regulation.

DISCUSSION

A number of chemicals, including some chemotherapeutic agents, have been demonstrated to induce erythroid differentiation of K562 cells, among which hemin is one of the most potent erythroid differentiation inducers (8, 59). During hemin-mediated erythroid differentiation of K562 cells, both transcriptional and translational upregulation of the ferritin gene was observed (42), resulting in an increase in ferritin synthesis and ultimately enhancing cellular capacity of iron storage and supply upon metabolic requirement for hemoglobinization (2, 45, 72). In contrast to well-elucidated translational regulation of ferritin synthesis by hemin (27, 40, 55, 57), molecular mechanisms by which transcription of the ferritin gene is regulated during hemin-induced erythroid differentiation have been elusive. Coccia and colleagues demonstrated that dimethyl sulfoxide or HMBA had minimum effect on ferritin H mRNA induction during erythroid differentiation of MEL cells but that hemin induced ferritin H at the transcriptional level (19, 20). In their further study, hemin induced NF-Y binding to a proximal CCAAT element by which transcription of the human ferritin H gene was activated (41). In our experiments using K562 human erythroleukemia cells, we

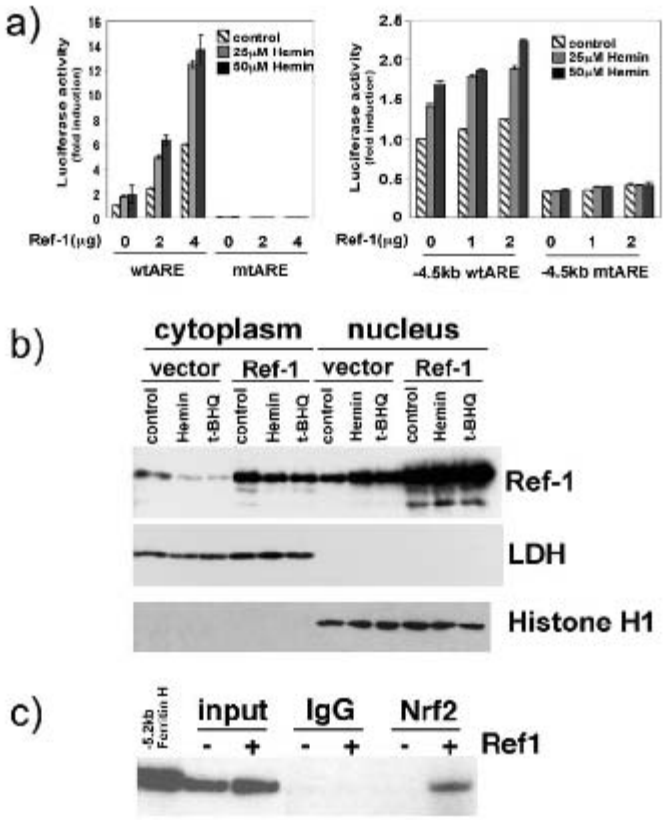

FIG. 8. Activation of the ferritin H ARE by Ref-1 and increased Ref-1 nuclear localization by hemin or t-BHQ treatment. (a) One microgram of ferritin H ARE-luciferase (left panel, the wild-type or double mutant ARE in the minimum promoter construct shown in Fig. 3b; right panel, −4.5kb ferritin H containing the wild-type or double mutant ARE shown in Fig. 3c) and 20 ng of pRL-EF together with 1 to 4 μg of pCMV-Ref1 expression vector were transfected into K562 cells by electroporation. Hemin (25 or 50 μM) was added 24 h after transfection, and cells were incubated for an additional 24 h. The cells were harvested to measure firefly and *Renilla* luciferase. The induction from the wild-type ARE transfectant (wtARE) without treatment was defined as 1.0. The results from three independent experiments are shown as means ± standard errors. (b) K562 cells transfected with 4 μg of pCMV-Ref1 or pCMV empty vector, followed by treatment with 50 μM hemin or 10 μM t-BHQ for 4 h were subjected to isolation of nuclear and cytoplasmic fractions. One microgram of nuclear protein and 6.5 μg of cytoplasmic protein were used for Western blotting with anti-Ref1 antibody. Anti-LDH and anti-histone H1 antibodies were used for verification of the purity of the fractions. (c) K562 cells (1×10^7) were transfected with 10 μg pCMVRef-1, and a ferritin H ARE ChIP assay was carried out to assess endogenous Nrf2 binding to the ARE. The −5.2kb ferritin H-luciferase plasmid was used as a PCR control and as a size marker of the 155-bp DNA fragment.

did not observe a significant role of the CCAAT element in hemin-mediated activation of the ferritin H gene (Fig. 2), perhaps due to the difference in cell lines and species; however, the region containing CCAAT indeed served as a potent proximal enhancer of the ferritin H gene in K562 cells (Fig. 2).

It was demonstrated that hemin- as well as anthracycline-mediated K562 erythroid differentiation was blocked by antioxidants, suggesting that oxidative stress is involved in a chemically induced erythroid differentiation process (18, 46). Furthermore, a Fenton reagent (Fe^{2+} plus H_2O_2) was shown to induce erythroid differentiation of K562 cells (47). These observations led us to hypothesize that hemin may activate transcription of the ferritin gene through an ARE enhancer as a result of hemin-mediated oxidative stress. We tested this hypothesis in this study and found that a hemin-responsive element for transcriptional activation of the human ferritin H gene is identical to the ARE (Fig. 2 and 3), which was also activated by t-BHQ, a potent ARE activator (38). Intriguingly, Beaumont et al. reported that a far-upstream 180-bp region of the mouse ferritin H gene contains a regulatory element for ferritin H transcriptional activation during differentiation of MEL cells with HMBA (7). We reviewed this far-upstream

180-bp region and confirmed that it in fact contains an ARE that we previously identified as an enhancer for transcriptional activation of the mouse ferritin H gene in response to oxidative stress (69, 70). Since the human and mouse ferritin H AREs are well conserved in their DNA sequences, including two essential AP1 motifs (68, 70), we speculated that activation mechanisms of the ARE may be shared in both human and mouse ferritin H genes during erythroid differentiation of K562 by hemin and MEL by HMBA. However, our preliminary experiments of transfection of either mouse or human ferritin H-luciferase reporters into MEL cells showed that HMBA did not activate the ferritin H ARE (our unpublished observations). In fact, Beaumont et al. (7) reported that a mutation in the NF-E2 site within the 180-bp region of the mouse ferritin H gene (which is in the ARE) (68) did not abolish the induction of ferritin H by HMBA treatment. Therefore, all experimental results available so far suggest that HMBA-mediated activation of the ferritin H transcription in MEL cells requires an unidentified enhancer element. Further investigations will be necessary to understand the molecular mechanism by which HMBA activates the transcription of the ferritin H gene during erythroid differentiation of MEL cells.

It should be noted that one of the AP1 motifs in the ferritin H ARE is identical to the NF-E2 binding site (Fig. 3). NF-E2, belonging to the b-zip transcription factor family, forms a heterodimer of p45NF-E2 and the small Maf family of transcription factors (3). NF-E2 is involved in transcriptional regulation of the β-globin gene through locus control regions (13). However, homozygous p45 NF-E2 knockout mice displayed relatively mild defects in erythroid development with normal β-globin expression levels (60), probably due to compensation of p45 NF-E2 function by other transcription factors. We could not obtain results to indicate the contribution of NF-E2 to ferritin H ARE activation in ARE-luciferase reporter assays and gel retardation assays or a hemin-mediated localization change of NF-E2 by Western blotting (data not shown). Although further investigation will be necessary, we currently understand that NF-E2 may not be a key regulator of ferritin H transcription during hemin-mediated erythroid differentiation of K562 cells. This is consistent with the observation by Beaumont et al. in which they did not find the contribution of NF-E2 protein to mouse ferritin H transcription during HMBA-induced erythroid differentiation of MEL cells (7).

Among AP1/Nrf b-zip transcription factor family members, our ChIP assays showed that hemin induced binding of cJun, JunD, FosB, and Nrf2 to the ferritin H ARE in K562 cells (Fig. 4). Although several groups demonstrated that induction of nuclear translocation or accumulation of Nrf2 is an activation mechanism of ARE by oxidative stressors (32, 35, 49) or hemin treatment (33), we did not see clear induction of nuclear translocation or accumulation of these transcription factors, including Nrf2 after hemin treatment in K562 cells (Fig. 7). We therefore speculated that posttranslational modifications of these transcription factors following hemin treatment may enhance their abilities of DNA binding. Ref-1, a redox factor of AP1, was previously demonstrated to reduce a conserved cysteine residue in the DNA binding domains of AP1 family members and enhances their binding to the AP1 binding sequence (1, 74). Ref-1 was also shown to be activated during oxidative stress, at least in part, by the mechanism of enhanced nuclear translocation of Ref-1 itself (4, 63) or thioredoxin (30, 34). In this study, we showed that Ref-1 is localized in both the nucleus and cytoplasm in K562 cells and that hemin as well as t-BHQ treatment induced nuclear accumulation of Ref-1 (Fig. 8b). Furthermore, Ref-1 activated the transcription of the ferritin H gene through the ARE (Fig. 8a). It should be noted that these results are consistent with those supporting the redox-dependent regulation of transcription factors bound to ARE (34) in addition to direct evidence showing that a mutation of cysteine 506 to serine in the mouse Nrf2, which is a potential Ref-1 site, significantly diminished Nrf2-mediated activation of ARE by t-BHQ (11).

Ferritin has been understood as a major cytoplasmic iron storage protein; however, a growing number of reports have demonstrated that ferritin H is also localized in the nucleus (14) as well as in mitochondria (5, 16, 21, 23, 37). Although the function of nuclear ferritin is not completely understood, it was reported that nuclear ferritin H protects DNA from UV- or iron-induced DNA damage (15, 66). Furthermore, human ferritin H but not ferritin L was shown to bind to DNA, with no clear preference for DNA sequence or the nature of DNA ends (62). In K562 cells, roles of nuclear ferritin in gene regulation have also been reported. The first observation was that ferritin H and L cDNAs were isolated by subtractive hybridization between hemin-treated and nontreated K562 cDNAs and that ferritin L had the greatest activation of the γ-globin reporter construct in transient transfection assays (73). Recently, it was reported that a ferritin family protein in K562 nuclear extracts binds specifically to the CAGTGC motif in the β-globin promoter at bp −153 to −148 (12). In the same study, recombinant ferritin H protein was shown to bind to this sequence in a sequence-specific manner, and transfection of the ferritin H expression plasmid into CV-1 cells repressed the β-globin promoter in reporter assays (12). On the other hand, results contradictory to these observations were also reported, in which the presence of ferritin in the nucleus of K562 cells was confirmed and an antiferritin antibody blocked protein binding to a β-globin promoter region; however, the protein was heat sensitive, with a molecular mass of 90 to 100 kDa, and also, recombinant ferritin H or L failed to bind to the β-globin promoter (53). In our study, t-BHQ treatment of K562 cells induced ferritin H synthesis but failed to induce hemoglobin synthesis as a hallmark of erythroid differentiation (our unpublished observations), suggesting that ferritin H induction may not be sufficient to alter, or may not be directly coupled with, transcriptional regulation of globin gene expression. However, induction of ferritin synthesis, and perhaps subsequent decrease in ferritin synthesis (remains to be elucidated), to donate maximum levels of stored iron in the ferritin shell for heme synthesis should be critical to cell maturation and completion of erythroid differentiation (45, 72).

Induction of ferritin H synthesis during erythroid differentiation appears to be important not only for storage of iron but also for cytoprotection against oxidative stress-mediated cytotoxicity. Increased ferritin content in the cells was shown to induce cytoprotection from oxidative damage (6, 17, 54). In addition, we and others demonstrated by overexpression that ferritin H is cytoprotective against oxidative stress in erythroleukemia cells (24) and other cell types (22, 50, 52). Interestingly, preincubation of human leukemia cells with hemin induced

cellular resistance to oxidants due to enhanced expression of ferritin H, with minimum contribution of ferritin L to the cytoprotective effect (39). Therefore, transcriptional activation of the ferritin H gene as well as other phase II detoxification genes, such as NQO1 and GST-pi (Fig. 6), through the ARE appears to be a reasonable cellular defense mechanism to adapt to subsequent alterations in iron metabolism and oxidative stress during hemin-mediated erythroid differentiation.

In this study, we have defined the molecular mechanism by which hemin regulates transcription of the human ferritin H gene during erythroid differentiation of K562 cells. While this paper was under review, Hintze and Theil demonstrated that a human ferritin L ARE is activated in HepG2 liver cells by hemin treatment (29), suggesting that both ferritin H and L subunits can be regulated by hemin through a similar ARE element during erythroid differentiation. The ARE-mediated mechanism of ferritin induction at the transcriptional level may be extrapolated to other prooxidative conditions, such as malignancies, infectious diseases, and inflammation, under which elevation of serum ferritin levels has been frequently observed.

ACKNOWLEDGMENTS

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